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THE EFFECT OF PROTEIN DEFICIENCY ON THE RESISTANCE OF RATS TO INFECTION WITH SPIROCHETES OF RELAPSING FEVER

K. GUGGENHEIM, E. BUECHLER-CZACZKES, AND S. HALEVY

From the Laboratory of Nutrition, Hebrew University-Hadassah Medical School, Jerusalem, Israel

The effect of protein deficiency on the resistance of rats and mice to infection with *Salmonella typhi murium* has been reported in previous publications from this laboratory.[1,2] Physicians in the Middle East[3] have often been impressed by the severe course and the high fatality rate of this infection in a malnourished population. We, therefore, undertook a study of the effect of protein deficiency—a form of malnutrition which is often encountered in this region—on the resistance of the host to relapsing fever spirochetes. This paper presents results obtained by the experimental infection of normal and protein deficient rats with spirochetes of relapsing fever.

METHODS

Male rats within one week after weaning were fed diets of either one of the following compositions:

	Standard diet	Protein deficient diet
Rice starch	73 g	91 g
Casein	18 g	
Olive oil	5 g	5 g
Salt mixture	4 g	4 g

One-tenth mg thiamine hydrochloride, 0.2 mg riboflavin, 5.0 mg nicotinic acid, 0.1 mg pyridoxin, 1.6 mg calcium pantothenate, 0.25 mg pteroylglutamic acid, and 100 mg choline chloride were incorporated into the diets. Each rat received 100 I.U. vitamin A and 4 I.U. vitamine D twice weekly.

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2. Guggenheim, K. and Buechler, E. 1948, J. Immunol. **58**: 133–139.
3. Wolman, M. Personal communication.

Ninety-six rats were divided into three groups: group A, 24 rats, received the standard diet, ad libitum; group B, 36 rats, was fed the protein deficient diet, ad libitum; and group C, 36 rats, was given the standard diet, restricted in amount to the level of intake of the protein deficient diet by group B.

After two weeks on these diets 24 rats of each group were infected with *Borrelia persica** by intraperitoneal injection of 0.3 ml of an emulsion prepared by grinding one brain of an infected rat in 5 ml citrate solution. Twelve rats of group B and C were not infected and served as control. The rats were observed for 3 weeks and samples of their blood were examined daily by a thick drop preparation stained with Giemsa. The degree of infection was estimated in the following way: 0—no spirochetes found in the preparation; 1—few spirochetes in the whole preparation; 2—one spirochete in every second to tenth microscopical field; 3—one spirochete in every field; 4—2 to 5 spirochetes in every field; 5—6 to 10 spirochetes in every field; 6—11 to 15 spirochetes in every field; 7—16 to 20 spirochetes in every field; etc.

RESULTS

Table 1 shows the weight changes and food consumption of the three groups of experimental rats. As can be seen from Table 1, food consumption and weight increases in group A were normal, whereas the protein deficient group B consumed considerably less food than group A and decreased in weight. Group C, pair-fed with group B but given standard diet, increased in weight to a small extent only.

During the first two days after the infection no spirochetes were found in

* The strain of *B. persica*, a variety encountered in the Middle East, was kindly provided by Prof. S. Adler, head of the Department of Parasitology of the Hebrew University.

TABLE 1.—*Food consumption and weight changes of rats fed different diets.*

Type of diet	Daily food consumption		Body weight in g	
	2 weeks before infection	During 3 weeks of infection	At beginning of experiment	At day of infection
Group A: standard (ad libitum)	9.0	10.8	40	73
Group B: protein deficient (ad libitum)	3.9	2.5	39	32
Group C: standard (restricted in am't.)	3.9	2.5	38	43

the blood of any of the rats. The incubation period ranged from 3 to 5 days, the means being 2.9 days in group A, 3.0 days in group B, and 3.2 days in group C. The difference in the incubation periods observed in the three dietetic groups proved to be statistically not significant (table 2).

The periods during which spirochetes were present in the circulating blood varied between 1 and 10 days with an average of 1.9 days in group A, 3.7 days in group B, and 2.3 days in group C. The differences observed between groups A and B and those between groups B and C were found to be statistically highly significant.

The number of attacks per week did not differ significantly in the three experimental groups. In group A 1.06 attacks occurred per week, in group B 1.27, and in group C 1.25. These results and their statistical evaluation are summarized in table 2.

Considerable differences were observed in the relative degree of infection, as can be seen from table 3. The mean degree of infection was found to be more severe in group B than in group A. It seems that the lowered resistance in the protein deficient group was due partly to the protein deficiency per se and partly to the diminished food intake of this group. This was indicated by the results obtained with group C which was given the standard diet (as group A) but in restricted amounts (as was eaten by group B). The degree of infection of this group was higher than that of group A and lower than that of group B. Statistical analysis of the mean differences between the degrees of infection in the three groups showed that they were highly significant.

The infection with *B. persica* was not fatal in rats fed the standard diet. A high fatality was, however, observed in the group fed a protein deficient diet. Table 4 shows that 20 out of 24 infected rats, kept on the protein deficient diet, died in the course of the experiment, whereas in the uninfected control group of 12 rats, only 2 died. In the pair-fed infected group, 2 out of 24 rats died, but no deaths occurred in the noninfected control group. Table 4 shows also

TABLE 2.—*Incubation time, duration of attacks, and number of attacks per week in rats infected with* B. persica *and fed the 3 types of diet.*

Group	Incubation time in days			Duration of attacks in days			Number of attacks per week		
	Mean	σ	E	Mean	σ	E	Mean	σ	E
A	2.9	0.38	0.08	1.9	1.1	0.14	1.06	0.45	0.08
B	3.0	0.69	0.14	3.7	2.3	0.31	1.27	0.49	0.10
C	3.2	0.69	0.14	2.3	1.7	0.20	1.25	0.56	0.11
P*: A/B			0.6			0.01			0.1
A/C			0.05			0.1			0.2
B/C			0.3			0.01			0.9

* P: Probability.

the expectation of life in the 5 groups of rats studied. In our calculation the expectation of life was limited to 36 days experimental age. Any rat living for a longer period was regarded as having died on the 36th day, since the use of the unlimited expectation of life gives undue weight to the relatively few rats that live for much longer periods. A similar procedure has been adopted by Greenwood et al[4] in their studies on experimental epidemics in mice. It can be seen from table 4, that the protein deficient infected rats had a considerably lower expectation of life than the noninfected rats on the same diet or even the infected rats on standard diet given ad libitum or in restricted amounts.

TABLE 3.—*Degree of infection with* B. persica *in the 3 groups of rats. Means and standard deviations.*

Day of infection	A	B	C
3	0.3±0.06	0.2±1.29	0.1±0.28
4	2.0±1.41	1.4±1.30	0.7±1.07
5	2.1±1.60	3.0±1.30	2.5±2.16
6	0.7±1.08	3.9±1.61	2.3±1.13
7	0.8±1.48	3.0±2.20	1.2±1.41
8	0.2±0.08	3.2±3.86	1.0±1.13
9	0.7±1.20	3.6±4.29	1.4±1.40
10	1.3±1.63	4.3±4.63	2.0±1.98
11	0.6±1.05	4.3±3.78	1.2±1.83
12	0.3±0.86	2.9±3.87	0.8±1.10
13	0.3±0.68	1.8±2.13	0.9±1.09
14	0.5±1.53	1.4±1.73	0.4±1.06
15	0.5±1.10	2.1±2.95	0.2±0.39
16	0.5±1.10	0.8±2.37	0.3±0.88
17	0.4±1.17	1.2±1.98	0.8±2.22
18	0.6±1.26	2.8±3.45	0.8±1.45
19	0.1±0.44	1.6±1.62	0.7±1.72
20	0	1.4±2.43	0.6±1.40
Mean	0.7±1.37	2.6±3.05	1.0±1.51
Standard error, E	0.06	0.17	0.07
P	A/B≷0.01	A/C≷0.01	B/C≷0.01

DISCUSSION

Our results show that protein deficiency induces marked changes in the response of rats to an intraperitoneal infection with relapsing fever spirochetes. The mean duration of each attack increases from 1.9 days to 3.7 days, and the mean degree of infection from 0.7 to 2.6. Moreover, the infection which is not fatal in well-nourished rats, results in a high mortality rate in protein deficient rats. The deleterious effect of protein deficiency, however, is not entirely due to the protein deficiency per se. An important role is to be ascribed to the reduced caloric intake concomitant to the dietary lack of protein. Caloric restriction alone (group C) prolongs the duration of attacks, increases their severity, and raises the fatality rate. But the effect of caloric restriction appears to be a minor one in comparison with the deleterious effect of protein deficiency. It is, however, noteworthy, that protein deficiency has no significant influence on the incubation time.

The protein deficient organism appears to differ from the normal one in its reduced ability to eliminate spirochetes from its blood as rapidly and as efficiently as the normal. However, it is worthy of mention that blood preparations of dying rats were often found to be negative. Even rats *in extremis* can, therefore, eliminate spirochetes from the circulating blood. Studies to elucidate the mechanism of the diminished resistance of protein deficient animals to relapsing fever spirochetes are in progress in this laboratory.

4. Greenwood, M., Hill, A. B., Topley, W. W. C. and Wilson, J. 1936, Med. Research Council Special Report Series No. 209, 204 pp.

TABLE 4.—*Mortality of noninfected rats and of rats infected with* B. persica *fed the 3 types of diets.*

Group	Infected	No. of rats	No. that died in 35 days on diet (20 days after infection)	Av. no. of days before death of rats that died	Expectation of life, limited to 36 days experimental age
A	+	24	0		35.5
B	+	24	20 (83%)	28.8	29.5
B	−	12	2 (17%)	34.0	35.1
C	+	24	2 (8%)	24.0	34.5
C	−	12	0		35.5

The effect of dietary deficiency of protein on the resistance to various types of helminthic, protozoan, and bacterial infection has already been described. According to Donaldson and Otto[5] protein deficiency interferes with acquired immunity to *Nippostrongylus muris*. It reduces the resistance of chicks to *Plasmodium lophurae*.[6] Guggenheim and Buechler[1,2] observed a marked reduction in the rate of resistance of rats and mice to an infection with *S. typhi murium* in states of protein deficiency. This lowered resistance could be explained by a diminution of the bactericidal power and, to a lesser degree, of the phagocytic activity of the body fluids. The role of protein in resistance to experimental tuberculosis in rats has recently been studied by Koerner et al.[7] With a high protein diet, tuberculosis was shown to be less extensive, life was much longer, the degree of the localization of the disease was better, and the extent of pneumonia less than with a low protein diet. The effect of protein deficiency on the immune response has extensively been studied by Cannon and coworkers.[8–10] All these reports are in accordance with our finding of a reduced resistance in protein deficient rats to an infection with relapsing fever spirochetes.

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SUMMARY

1. Three groups of young rats were placed on the following diets: a full diet, given ad libitum; a protein deficient diet, given ad libitum; a full diet, restricted in amount to the level of intake of the protein deficient diet by the second group. After two weeks on these diets the rats were inoculated intraperitoneally with *Borrelia persica* and observed for three weeks.

2. Protein deficiency resulted in a more severe degree of infection, a longer duration of attacks, and a high fatality rate. There was no effect on the incubation time or on the number of attacks per week.

3. This diminished resistance of protein deficient rats was only to a minor degree the result of the concomitant caloric deficiency, but to a greater degree found to be the effect of protein deficiency per se.

HUMAN CARRIER OF THE PSITTACOSIS VIRUS

K. F. MEYER AND B. EDDIE

From the George Williams Hooper Foundation, University of California, San Francisco 22, California

The carrier and shedder stage so common in spontaneous psittacosis of many species of birds and readily produced by inoculation of mammalian and avian hosts has not been recognized in man with certainty, although the observations reported by Gerlach as early as 1936 suggested such a condition. The Austrian investigator had isolated the psittacosis agent from the sputum of five patients without clinical symptoms of parrot fever.

The examination of a patient with a persistent cough led to the isolation of the psittacosis virus from the respiratory discharges 8 and probably 10 years after a severe acute attack. This unusual opportunity was chosen to make a detailed study of the infective agent. With the methods now available it was not possible to demonstrate that the strain acquired characteristics which would place it into the human pneumonitis group. Antigenically, it is related to the meningopneumonitis agent.

CLINICAL HISTORY

The patient was a 36 year old biochemist whose laboratory was adjacent to another in which psittacosis virus studies were in progress. On April 10, 1938 he entered the hospital because of progressing illness which had begun quite suddenly 3 days before. His symptoms were those of a generalized infection—chills and fever, severe frontal headache, weakness, restlessness, abdominal pains and epigastric tenderness. Because of the possible exposure to equipment contaminated with psittacosis material and the nature of the disease, it was suspected that he had psittacosis. Positive diagnosis was made when the results of laboratory tests were available.

In general, the course (represented in fig. 1) was characteristic of that of severe psittacosis, including enlargement of the spleen. His cough, which was typically dry at first, later became productive. He had gastrointestinal symptoms—abdominal distention, pain and vomiting—and central nervous system symptoms—restlessness, delirium, nuchal rigidity and muscle twitchings.

An indication of early pulmonary involvement was apparent on the day of his admission to the hospital, impairment of resonance over the base of the left lung with increased bronchial sounds. By the third day, there were definite signs of pneumonia in the upper lobe of the left lung. On the 6th day it was necessary to put the patient in an oxygen tent. Three days later both lungs were involved. After treatment with a total of 125 ml of human serum containing neutralizing and complement-fixing antibodies in low dilutions on the 12th, 14th and 15th days, his temperature dropped, and the pneumonia began to subside. Resolution of the infiltration at the base of the left lung was rather slow, and recovery was complicated by cystitis. He was hospitalized for 45 days.

His cough, occasionally violent and paroxysmal, persisted, and in the succeeding 4 years he frequently had severe coughing spells, particularly early in the morning. These paroxysmal coughing spasms were productive of a considerable amount of thick tenacious sputum, full of pus cells and mucus.

From 1943 to 1946 this man served in the Armed Forces, first on this continent, then in Europe. On his return from duty abroad early in 1946, he was first assigned to a military hospital in California. During a visit at the Hooper Foundation, he was again seized with a violent coughing spell, and a specimen of sputum was collected. An agent of the psittacosis group was isolated from this specimem. A series of daily specimens was taken, and virus was recovered repeatedly. When these findings were reported to his station hospital, a careful complete examination was carried out, and penicillin therapy was instituted. Repeated X-ray examinations in June 1946, revealed a normal lung field and sinuses. No anatomical lesions responsible for the paroxysms of coughing could be found. The tuberculin, the Kahn and the Frei tests were all negative, but the complement-fixation reaction and the results of examination of the sputum were positive for psittacosis.

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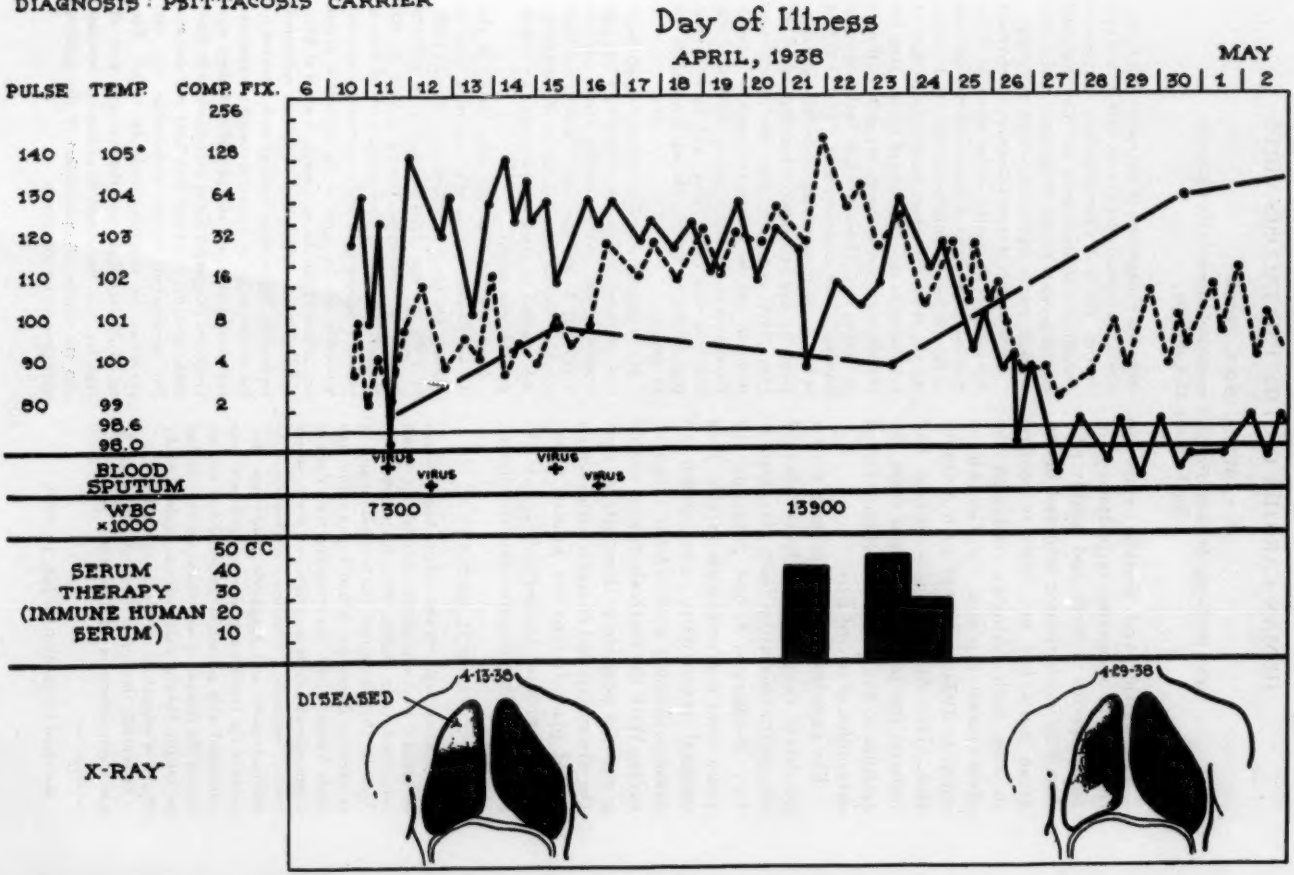
PATIENT: A.M.
AGE: 36
DIAGNOSIS: PSITTACOSIS CARRIER



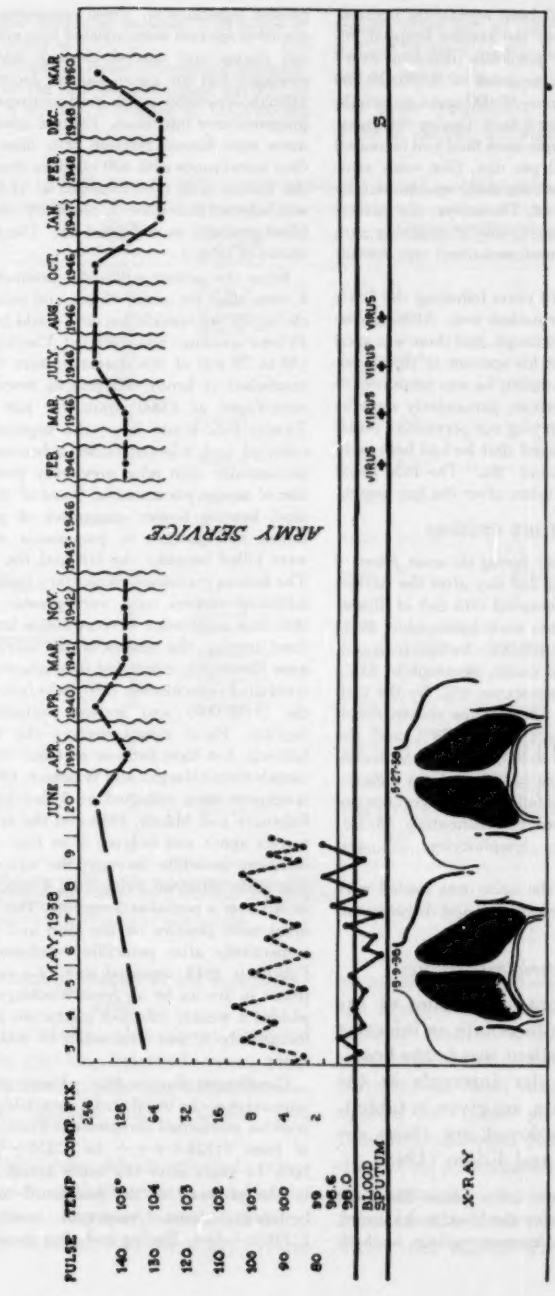


FIG. 1.—Clinical and laboratory findings in psittacosis carrier.

After the virus had been repeatedly isolated, the patient received at the station hospital, intensive intramuscular penicillin treatment every 3 hours for 10 days, (a total of 8,000,000,000 units). He was also given 50,000 units by nebulization every 3 hours for 8 days. During this treatment his sputum became more fluid and increased in amount to 250 ml per day. One week after treatment was stopped, the daily sputum output dropped to 50 to 70 ml. Thereafter, the patient noted less frequent paroxysms of coughing and, under thyroid treatment, remarked that he felt less tired.

In general, in the 10 years following the acute illness, the patient remained well. Although he often had a productive cough, and there was good reason to believe that his sputum at times contained the psittacosis agent, he was permitted to carry on normal activities, particularly since he was cooperative in carrying out preventive measures. In 1950 he reported that he had been well, except for two attacks of "flu." The 1950 blood serum specimen was taken after the last attack.

LABORATORY STUDIES

Routine examinations during the acute phase.—On April 11, 1938, the 2nd day after the patient was admitted to the hospital (5th day of illness) the results of blood tests were: hemoglobin, 89%; erythrocyte count, 5,400,000; leukocyte count, 7,300; differential cell count, neutrophils, 81%, lymphocytes, 13%, monocytes, 6%. By the 12th day, the picture had altered—the concentration of hemoglobin had dropped to 79% and the erythrocyte count to 4,200,000; but the leukocyte count had risen to 13,900, and the differential count revealed the following changes: neutrophils, 91% (filamentous/nonfilamentous, 55/36); metamonocytes, 3%; lymphocytes, 2% and monocytes, 2%.

On the 37th day, the urine was loaded with leukocytes and *Escherichia coli* and *Streptococcus viridans*.

Special bacteriologic studies

Results of laboratory studies of the blood and sputum to isolate an infective agent while the patient was in the hospital, and at irregular intervals in the subsequent 12 years, are given in table 1. The methods employed are those detailed by Meyer and Eddie (1948).

Sputum.—During the acute phase the agent was readily isolated from the blood and sputum, but during the convalescence various methods proved unsuccessful. Fresh specimens of mucupurulent sputum were injected into mice by various routes and carried through several blind passages, but the agent was not recovered. The animals invariably succumbed to streptococcic or pneumococcic infections. Diluted sputum specimens were filtered through Seitz filters or collodion membranes with 600 mμ pore diameter, and the filtrate after centrifugation at 15,000 r.p.m., was injected into mice. If necessary, several such blind passages were carried out. The results are shown in table 1.

Since the patient still had a productive cough 8 years after the initial illness, and neither tubercle bacilli nor specific bacteria could be found, a 24 hour specimen was collected. Varying amounts (30 to 70 ml) of this specimen were thoroughly emulsified in broth, refrigerated overnight, and centrifuged at 8,000 r.p.m. for half an hour. Twenty-four hours later, the supernatant was cultured and injected either subcutaneously or intranasally into mice previously proved to be free of mouse pneumonitis. Some of the animals died, bearing lesions suggestive of psittacosis; others had patches of pneumonia when they were killed between the 8th and the 10th day. The lesions contained elementary bodies, but in addition, various cocci were present. Since by this time antibiotics were available for more refined testing, the tissues of the sacrificed mice were thoroughly emulsified in a mixture of streptomycin hydrochloride (250 units/ml), tyrothricin (1:50,000) and sodium sulfadiazine (50 mg/ml). These agents destroy the interfering bacteria, but have little or no effect on the psittacosis virus (Morgan and Wiseman, 1946). Seven specimens were collected at 8 day intervals in February and March, 1946 and the typical psittacosis agent was isolated from four. Following intensive penicillin therapy, the infective agent was again obtained twice from 4 specimens collected over a period of 7 months. The two specimens were positive on the 40th and 60th days respectively after penicillin treatment. During February, 1948, repeated tests of a sample sent frozen in dry ice by air from Washington, D. C., yielded a weakly infective psittacosis agent. Unfortunately it was impossible to maintain this agent.

Complement fixation tests.—Every year, re-examination of the blood serum invariably yielded a positive psittacosis complement-fixation reaction of from 1:128++++ to 1:256++++. In fact, 12 years after the acute attack, the titer in the presence of the psittacosis and of the lymphogranuloma venereum antigen was 1:256++++. During and after penicillin ther-

apy, the titer fluctuated between 1:64++++ and 1:256+++.

The neutralization index (Rivers and Schwentker, 1934) on the 75th day after onset was 9.5; on the 170th, it was 6.5; it dropped to −1.4 by the 10th year.

nasal, 0.03 ml of $10;^{-4}$ intramuscular, 0.5 ml of 10^{-2} and subcutaneous, 0.5 ml of $10.^{-4}$ On intraperitoneal injection, the mice frequently succumbed within 3 to 8 days; at autopsy, there were

TABLE 1.—*Special laboratory studies of a human carrier of psittacosis.*

Date	Time after onset	Isolation of virus	Complement fixation		Neutralization index*
			Psittacosis	Lymphogranuloma	
1938	Days				
Apr. 11	6	Blood +	1:2+++		
Apr. 12	7	Sputum +			
Apr. 15	9	Blood +	1:8++++		
Apr. 16	10	Sputum +			
Apr. 23	19		1:4+++		
Apr. 30	24	−	1:64++++		
June 20	75	−	1:256++++		9.5
Sept. 24	170		1:256++++	1:256++++	6.5
1939	Years				
Apr.	1	Sputum −	1:128++++		
1940					
Apr.	2	Sputum −	1:256++++		
1941					
Mar.	3	Sputum −	1:128++++		
1942					
Nov.	4	Sputum −	1:128++++		
1946					
Jan. 31	7		1:256++++	1:128++++	
Feb. and Mar.	7	4 of 7 sputums +			
May 2	8	Sputum −			
June 6 (10 days after penicillin)	8	Sputum −			
Jul. 6	8	Sputum +	1:256++++	1:128++++	
Jul. 27	8	Sputum +			
Aug. 19	8	Sputum −			
Sept. 14	8	Sputum −			
Oct. 30	8	Sputum S†	1:256++++	1:128++++	
1947					
Jan. 27	8		1:128++++	1:64++++	
1948					
Feb. 2	8		1:64++++	1:64++++	
Feb. 10	8	Sputum −			
Dec. 14	9	Sputum S	1:64++++	1:64++++	
1950					
Mar. 11	10		1:256++++	1:256++++	−1.4

++++ Strongly positive (100% inhibition of hemolysis).
+++ Moderately positive (90% inhibition of hemolysis).
* Rivers and Schwentker method.
† Virus suspected, interference by paratyphoid.

Experimental studies of the infective agent

Experimentally, this agent isolated from the carrier, infected mice, ricebirds, parakeets, pigeons, housefinches, sparrows, guinea pigs, rabbits and monkeys.

The dilutions which caused infection in mice, and the routes of administration were: intraperitoneal, 0.5 ml of $10;^{-7}$ intracranial, 0.01 ml of $10;^{-4}$ intra-

areas of focal necrosis in the liver, and the spleen was enlarged. The elementary bodies, readily demonstrable in the plastic peritoneal exudate, were tinctorially, as well as morphologically, typical of those of the psittacosis-lymphogranuloma group. Infections induced by subcutaneous, intramuscular or intranasal route were irregularly fatal in the lower dilutions only, but either the spleen was enlarged or the

liver was enlarged, with marginal necrosis, or covered with a gray plastic exudate. There were characteristic grayish consolidations of the lungs of intranasally infected mice sacrificed on the 20th day.

Two ricebirds (*Munia oryzivora*) and 3 parakeets died 14 to 18 days after intramuscular injection of 0.2 ml of a 10% suspension of mouse spleen infected with the agent. The serofibrinous plastic deposits over the pericardium, air sacs, and liver and the splenomegaly were typical of psittacosis.

Pigeons whose serum had given negative complement-fixation reactions for psittacosis resisted intramuscular or intraperitoneal injection of the virus, but complement-fixing antibodies developed. In one of 5 pigeons given subdural injections, fatal meningitis developed.

Two of three housefinches and one of two sparrows died of the infection after intramuscular injection of a suspension of the agent.

When a suspension was injected intracranially into guinea pigs, they became paralyzed temporarily, but ultimately recovered. Guinea pigs were given 0.5 ml of a 10% solution containing the agent intraperitoneally. Fever developed, but none of the animals died. Intratracheal infection was fatal to all 5 animals given the agent by this route.

Cross-protection tests in guinea pigs.—The relationship of the Mu46 strain was compared with the Louisiana(Borg) strain through the cross-protection tests on guinea pigs. In the first study, 8 guinea pigs were inoculated intraperitoneally with 1 ml of a highly concentrated suspension of mouse spleen containing the Mu46 virus. They had fever for 2 to 4 days and lost weight. Serum collected on the 30th day after injection gave positive complement fixation reactions in varying dilutions; the titers varied from negative to 1:32. The serum of 5 reacted with the psittacosis antigen only, that of 2 with both the psittacosis and the lymphogranuloma antigen and that of 1 did not react with either. Seven of the 8 were then challenged on the 31st day by intraperitoneal injection of 0.5 ml of a yolk sac suspension of the Louisiana virus. These 7 were immune to this infection. When they were sacrificed on the 40th day, virus could not be recovered from pooled spleen and liver. The complement fixation titers at the time the experiment was terminated varied from 1:2++++ to 1:256++++; the titer of one was higher than 1:256++++. In the control series, the Louisiana virus had killed 6 of 10 guinea pigs. They had lesions characteristic of those of psittacosis—enlarged spleen, hemorrhages in the adrenals and serous membranes of the viscera, and exudate in the peritoneal cavity.

Forty-five animals were used in a second cross-protection test; 15 were inoculated and 30 served as controls. The 15 were given intraperitoneal injections of 0.5 ml of an undiluted yolk sac suspension of the Mu46 agent. One died on the 6th day from an intercurrent streptococcic infection and 4 others were sacrificed on the 48th day after the injection. The complement fixation titers of these 4 varied between 1:4++++ and 1:16++++. When these 5 animals were autopsied, virus was not demonstrable in the organs. On the 49th day, the remaining 10 guinea pigs were challenged intraperitoneally with a yolk sac suspension of the Louisiana virus. Three died from shock within 12 to 24 hours and 3 from psittacotic infection (7th, 11th and 12th days). Twenty-eight days after challenge, the 4 surviving animals (57% of the 7 animals that survived the initial phase) had a complement fixation titer of

1:256++++. Among the unimmunized controls given Louisiana virus, 12 of 30 (40%) survived.

Cross-protection tests in mice.—Groups of 100 mice were immunized by a single subcutaneous inoculation of 0.5 ml of yolk sac suspension of living organisms. The carrier strain, Mu46, was administered in a dose of 0.5 ml of a dilution of 10^{-5} and the meningopneumonitis (Francis) strain in a dose of 0.5 ml of a dilution of 10^{-2}. Three weeks after inoculation, groups of treated mice were challenged by the intracerebral route with 0.03 ml of the infective agent diluted in broth. Approximately 10,000 MLD of previously titrated suspensions of the four agents of different origin were inoculated. The meningopneumonitis strain had been obtained from Dr. Thomas Francis, Jr; the ornithosis strain 207 had been isolated from the pericardial exudate of a pigeon and the mouse pneumonitis virus from a mouse dead on arrival at the laboratory from Bar Harbor, Maine. This strain resembles the mouse agent described by de Burgh, Jackson and Williams (1945). The results of two experimental series are summarized in table 2.

TABLE 2.—*Results of cross-protection tests with mice. Mu46 and meningopneumonitis viral agents.*

Immunizing strain*	Challenging strain†							
	Meningopneumonitis (Francis)		Ornithosis (207)		Mouse pneumonitis (Bar Harbor)		Mu46	
	S/T	% S	S/T	% S	S/T	% S	S/T	% S
Mu46	12/18	67	11/16	69	14/18	78	9/15	60
	16/18	89	16/18	89	16/18	89	16/18	89
Meningopneumonitis (Francis)	17/18	94	16/18	89	18/18	100	15/17	88
	16/18	89	16/18	89	17/18	94	15/18	83
Controls	0/20	0	0/20	0	0/20	0	0/20	0

* Given subcutaneously.
† 0.03 ml (10,000 MLD), given intracerebrally.
S = Survived.
T = Total mice.

The carrier strain induced considerable resistance against the three strains, provided the immunity against the homologous challenge was of a high order. Meningopneumonitis virus immunization protected well against the ornithosis (Bar Harbor) and mouse pneumonitis agents, but less so against the carrier strain Mu46. These tests merely indicate that the Mu46 agent has a broad antigenic pattern of considerable antigenicity. Moreover, the results are in accord with similar cross-protection tests which in recent years have been employed to study the interrelationship of agents belonging to the psittacosis-lymphogranuloma group (Beck et al, 1944; de Gara and Furth, 1948; Wagner et al, 1949).

The experimental series demonstrated that the living Mu46 strain in a mouse-spleen suspension protected guinea pigs against a challenge infection with the Louisiana pneumonitis agent in a dose fatal to 60% of the nonimmunized animals. In the second series, the immunizing Mu46 agent prepared in the yolk sac sensitized, and the challenge injection with the identical material caused death by shock. Moreover, several animals suffered from latent streptococcic infections.

The challenge infection which caused death of 60% of the animals also fatally infected three of 7 guinea pigs; the resistance of the guinea pigs vaccinated with Mu46 was incomplete. However, it is in part significant that in experiments by Larson and Olson (1946), killed psittacosis and meningopneu-

monitis antigens in repeated doses conferred partial (40% to 60%) protection against the Louisiana agent.

Toxin.—In a detailed study of the endotoxins of viruses of the psittacosis-lymphogranuloma group, Manire and Meyer (1950) found that dilutions of the Mu46 strain grown in the yolk sac of embryonated hens' eggs fatally intoxicated mice on intravenous injection. In experiments in which aureomycin was used to study these viruses, the effect of the endotoxin was conclusively demonstrable in mice under prophylactic treatment with this antibiotic. Approximately 43% of the untreated mice and approximately 20% of the treated succumbed to the toxin within 24 to 48 hours after injection.

TABLE 3.—*Neutralization of psittacosis agents by Mu46 antiserum.**

Virus	Neutralization, LD_{50}
Mu46	11†
Meningopneumonitis	100
Parakeet strains	
6BC	10
Pasadena	2
Detroit	1
Duck strains	
Petaluma	1
Long Island	1
Pigeon strains	
Endo 3	6
Casano	10
Human pneumonitis	
Louisiana	3
Seaton	7
Friend	3
Gardner	19

* Prepared with roosters, given intracerebrally.
† In 5 tests.

The toxin of the Mu46 virus, which may be considered as moderately active, is not neutralized by chicken antitoxic serum prepared with the Louisiana (Borg) or the San Francisco pneumonitis viruses. On the other hand, the antiserum in chickens prepared with the toxin of strain Mu46 neutralized the toxin produced by the Illinois (Zichis and Shaughnessy) and the San Francisco pneumonitis, several human psittacosis, pigeon and duck agents. However, it failed to neutralize the Louisiana (Borg) or the feline pneumonitis toxins.

Neutralization tests with antiserum.—Incidental to a detailed study of the value of the neutralization test of Hilleman (1945), St. John and Gordon (1947) and Eddie et al (1950), more was learned about the antigenic relationship of the Mu46 agent to other viruses of the psittacosis-lymphogranuloma group.

Immune serum was prepared by inoculating roosters twice a week for 21 weeks with large doses of living Mu46 virus grown in the yolk sac. Twenty-one days after the last injection, the birds were exsanguinated; the serum was stored in the refrigerator at −4C. Mixtures of serum and descending dilutions of different agents of the psittacosis group available at the laboratory were made, and after incubation for two hours, were inoculated intracerebrally into a series of 10 mice each. Rather extensive experience had taught that the method possesses limited value in the differentiation of the avian psittacosis strains. Despite the selection of roosters serologically noninfected with ornithotic agents, it has been impossible to produce antiserums with high neutralization titer. The Mu46 antiserum, which neutralized 11 LD_{50} units of the homologous agent, however, neutralized 100 LD_{50} units of the meningopneumonitis virus (table 3). It likewise neutralized 10 LD_{50} units of an agent isolated from the sputum of a patient exposed to infected pigeons, and 19 LD_{50} units of an agent found at autopsy in the lung tissues in a case of fatal pneumonitis. The source of this infection could not be determined. The Mu46 serum exerted little or no protection against three other human pneumonitis, two parakeet, one pigeon and two duck strains.

For comparison, 8 antiserums which neutralized homologous psittacosis viruses in varying degrees were employed in neutralization tests with Mu46. The serum-virus dilution mixtures were tested both by intracerebral and intranasal inoculation of mice. The data presented in table 4 represent the averages of two separate tests.

Of two antiserums capable of neutralizing over 100 LD_{50} units of the homologous agent, one inactivated an equal number of LD_{50} units of the Mu46 viral agent. The strain with which this antiserum was originally prepared was isolated from the consolidated lung of a C57 mouse that had died en route between Bar Harbor, Maine and San Francisco in 1942. The antigenic relationship of the carrier strain Mu46 to the meningopneumonitis viral agent is reflected by the neutralization of 21 LD_{50} units by a moderately potent serum. Antiserum prepared with pigeon and parakeet ornithosis and mouse and human pneumonitis agents neutralized insignificant units of the Mu46 virus.

TABLE 4.—*Neutralization of Mu46 agent by various psittacosis antiserums.*

Antiserum*	Neutralization, LD_{50}		
	Viral agent		
	Homologous	Mu46	
	Intracerebral	Intracerebral	Intranasal
Pigeon			
207	40	10	
Endo 3	76	3	
Parakeet			
6BC	4	8†	19
Detroit	3	4	7
Pasadena	3	40	12
Mouse pneumonitis			
Greb	—	1	
Bar Harbor	134	130	
Meningopneumonitis			
Francis	145	21	
Human pneumonitis			
San Francisco	27	3	
Mu46	11	11	7

* Prepared with roosters.
† In 2 tests.

Summary of laboratory studies of the viral agent isolated from the carrier

Unfortunately, the psittacosis strain (Maine) of parakeet origin suspected as the cause of the infection herein reported, and the strains isolated from the blood and sputum of the patient in 1938 were lost. Therefore, the antigen of the strain when it caused the original infection could not be compared with that of the carrier stage obtained 8 years later.

The endotoxin of the Mu46 strain isolated in 1946 has an antigenic spectrum common to many psittacosis agents, but not to the Louisiana (Borg) and feline pneumonitis agents. Immune serum prepared on roosters with living Mu46 neutralizes only the meningopneumonitis agent. The mouse pneumonitis (Bar Harbor), meningopneumonitis and ornithosis (pigeon 207) antiserum neutralize significant Mu46 agent units. Intracerebral cross-protection tests of mice infected by the subcutaneous route in part confirm the antigenic relationship of the carrier strain to the meningopneumonitis virus. In guinea pigs the Mu46 agent induced considerable resistance against the Louisiana pneumonitis agent. Antigenically related to the meningopneumonitis virus, the Mu46 agent, on the basis of intraperitoneal virulence for mice and intracerebral virulence for pigeons, has retained certain characteristics of the original parakeet virus responsible for the infection and subsequent carrier stage.

EPIDEMIOLOGIC OBSERVATIONS

An infective agent in dynamic balance with its natural bird or mammalian host may likewise be biologically endowed with the ability to vegetate as a parasite in a latent stage in an aberrant host. The possibility that the patient might be a carrier and possibly a shedder of the psittacosis virus was considered as early as 1939, even though examinations of his sputum made at that

time did not reveal the virus, largely because of the high virulence of the concomitant bacterial flora of the respiratory tract.

When a mild psittacosis infection was diagnosed in one of the patient's associates, an elderly laboratory technician, the possibility was again carefully appraised. This woman worked in a room adjacent to the laboratory where the patient had occasional paroxysms of coughing. However, it should be noted that the technician had additional opportunities for exposure. She worked near the centrifuge suspected as the most likely source of infection of our patient. Still other insidious sources of infection are suspected, because she cleaned all of the glass culture flasks used for the mass cultivation of the psittacosis agent but supposedly sterilized before transfer to the washroom. The attack of the laboratory technician was relatively mild. Her illness began with a severe headache and fever. When definite rales developed at the base of the right lung, a tentative diagnosis of psittacosis was made. An agent was not isolated from the sputum, but her complement fixation titer rose from 1:2 to 1:16 by the time she returned to work on the 21st day after the onset of the illness.

From 1938 to 1943 the patient carrier was daily in close contact with at least 20 to 30 susceptible persons, both in the laboratory building and in the hospital. Eight persons were constantly exposed to the aerial droplet nuclei of his coughing spells, without consequence. They were under close scrutiny during their exposure; their serum was routinely examined for specific psittacosis antibodies at frequent intervals. Aside from the mentioned technician, clinical or latent psittacosis infections were not encountered during the 5 years the patient worked in that laboratory.

While he was in the armed forces, and therefore entirely independent of his presence, a small epidemic of at least 5 psittacosis infections occurred in the building where he had worked, because the rules for decontamination of highly infected egg material were broken. According to available reports and searching inquiries at various Army hospital laboratories, he is not suspected as the source of any pneumonitis infections.

It was of considerable epidemiologic importance that in November, 1946, 4 months after the agent was isolated from his sputum, he married. Since he was fully familiar with the significance of his infection, he paid particular attention to possible risks to which he exposed his wife. Shortly after his marriage, he left for the eastern part of the United States and, hence, was no longer under our observation. However, he has sent back frequent reports. His wife remained well, except for a stubborn cold with fever and little cough, 11 months after their marriage. Her blood serum tested at that time did not fix complement in the presence of the psittacosis antigen. In fact, repeated tests of her serum from 1946 to 1948 invariably proved negative, while those of her husband's serum continued to be positive. There is no evidence that she contracted psittacosis.

In view of these epidemiologic observations, it is reasonable to conclude that the infectivity of this psittacosis carrier is of a very low order. The laboratory examinations indeed support these conclusions. As might be expected from analogous infections, the amount of virus present in a 24 hour pooled sputum specimen was inconstant; it was rarely present in large amounts and was of relatively low pathogenicity for mammals.

After 1946 it became technically very difficult to secure suitable sputum specimens because the patient's coughing spells definitely diminished in frequency and intensity. Moreover, the concentration of serum antibodies adjusted to a lower level, and the probability of ultimate complete recovery from the carrier stage was anticipated. However, a retest of the patient's serum in 1950 rudely contradicted this optimism. The serum antibody level had returned to 1:256 and he complained of frequent spells of malaise.

The element of chance always plays a role in the heterogeneous infection chains. One realizes the probability that under appropriate circumstances the patient might initiate a human infection. Fortunately the circumstances of this infection have been conducive to its control. The patient was taught the necessary preventive measures and has been co-operative through this long period. Since he exerts scrupulous precautions while coughing and is careful in disposing of his sputum, his activities have not been limited.

DISCUSSION

Even presumptive evidence that human beings may harbor and spread the psittacosis agent is scarce, and the carrier state has been the subject of little systematic investigation. Unfortunately good opportunities for study have been missed, either because investigators did not have it in mind when the cases were encountered or because questionable motives have controlled investigations. Information therefore must be gleaned

from occasional reports in which this was not a point of special interest. In view of this limited information, it seems likely that human psittacosis, as many other diseases, may be spread either through subclinical latent infections or postconvalescent carriers.

There was a suggestion as early as 1930 that the infection might remain subacute, when Sturdee and Scott observed that persons in the bird-fancying trade have an apparent immunity to psittacosis. The nature of the disease has, of course, focussed attention on this group and it has since been found that persons who handle infected birds or who live in the vicinity of aviaries or zoological gardens not infrequently have significant levels of complement-fixing antibodies (Meyer and Eddie, 1939; Meyer, 1942). This group did not have demonstrable respiratory disease, and it was therefore thought that attempts to isolate the virus from the sputum would be futile. More information about subacute infection in this group might be gained under these circumstances if throat washings or respiratory secretions were secured, with the aid of expectorants if necessary, and studied by the mouse-inoculation technique.

The pathologic findings in the disease point to the lungs and to the spleen and liver as possible sites in which the virus could find conditions suitable for prolonged activity within the host. In the former, the danger of man-to-man transmission is obvious, while in the latter, the carrier state would likely remain "closed." Systematic inoculation of mice with pneumonic or splenic tissues obtained at routine autopsies on human beings has occasionally led to isolation of a psittacosis agent, though the clinical course did not suggest this infection (Meyer et al, 1942). In this connection the observations of Haagen and Krückeberg (1937) in Hamburg bear repetition and reconsideration. The sputum of one of their patients repeatedly contained the infective agent, but after the patient died of pulmonary embolism two weeks after convalescence from the psittacosis attack, only the spleen, not the lung, proved infective. On the other hand, it is known that the highly adapted mouse pneumonitis virus persists only in lung tissue. It is reasonable to accept the conclusions that persistence is accompanied by high serum complement fixation titers in carriers.

Five cases of latent psittacosis infection developed in the wards of the Vienna Krankenhaus at a time when patients with the clinical disease were under treatment (Gerlach, 1936). Intraperitoneal inoculation of sputum specimens collected from these patients produced disease in mice, and the microscopic findings at autopsy of the animals were characteristic of psittacosis. One of the 5 patients with the subclinical infection had mild symptoms, while the other 4 had none which could be attributed to psittacosis. Two of these patients whose sputum specimens were infective for mice were bed neighbors of patients with clinical psittacosis, while two others occupied in succession a bed previously occupied by a patient who had had a mild infection. According to Gerlach, infected birds could not justifiably be accused in these 5 cases.

As in the case reported herein, the infectivity of the agent in Gerlach's cases varied from that observed by others. It was necessary to passage it serially through 6 sets of animals over a period of 6 weeks before it produced recognizable elementary bodies. Surprisingly, inoculation experiments were not complicated by concomitant bacterial infections. Nor was this outbreak clinically similar to that observed in Hamburg (Hegler, 1929), Potsdam

(Hamel, 1932), Buenos Aires (Pinero García, 1940), Louisiana (Olson and Treuting, 1944) or in Zurich (Schmidt and Frei, 1931). Persons who became infected in those places through contact with psittacosis patients acquired manifest clinical symptoms. It is a little difficult to comprehend why none of the 5 contacts stigmatized as virus shedders in Gerlach's cases had clinically recognizable psittacotic symptoms during the entire critical period. His cases vary in still a third respect: with few exceptions in other reports, extreme contagion characterized only the fatal cases. In the Louisiana series, for instance, only those who had initmate contact with a fatal infection in the last 48 hours of illness contracted the disease (Olson and Treuting, 1944).

The ready demonstration of the psittacosis agent in the blood 72 days and in the sputum 75 days after the diagnosis of psittacosis in one of Gerlach's patients with chronic pulmonary tuberculosis after thoracoplasty has not aroused the curiosity it deserves. That the psittacosis agents circulate in the blood and can be demonstrated there until the 15th day is fully established (Meyer, 1948). On the other hand, it is biologically reasonable to suspect that in cases of tuberculosis accompanied by latent psittacosis of the respiratory tract, occasionally cells loaded with virus particles are thrown into the circulation as late as the 72nd day after diagnosis. In Gerlach's case there are no data concerning the preservation of the blood—whether it was defibrinated or citrated. Furthermore, the amount of blood inoculated into the mice is not mentioned.

Gerlach's findings are of considerable interest because they seem to illustrate that (a) human psittacosis is not invariably accompanied by recognizable clinical signs and symptoms or evidence of severe illness, (b) human beings may be subject to subclinical, latent psittacosis and be carriers and shedders and (c) man-to-man infections occur more frequently than is generally thought. If these be true, then the handlers of parrots, parakeets, pigeons, or other birds subject to the infection resume a focal point of interest as possible carriers. Such observations deserve confirmation particularly in view of the epidemiologic implications and extension on a broader basis.

After it was recognized that the psittacosis agent has a tendency to latent persistence in the spleen, liver and kidneys of mice and of parakeets (Meyer and Eddie, 1933), several authors—Fortner (1936), Haagen and Mauer (1938) and Doerr (1944)—discussed the probability that patients convalescent from clinical psittacosis may serve as healthy carriers, and for varying lengths of time, shed the agent without renewed exposure to the natural reservoir. Although evidence before 1946 failed to give undisputed support to this idea, something can be learned of the probable carrier stage through critical appraisal of the reports of Hoge (1934), Pfaffenberg (1936), Vervoort and Ruys (1939/40) and Haagen and Mauer (1938).

Observations on a patient who contracted psittacosis through exposure to infected parakeets (Pfaffenberg, fig. 10, p. 275) disclosed the important facts that (1) the findings of the agent in the sputum were irregular—either negative or positive—in the course of the illness and (2) the infective agent was discharged in fairly large amounts, apparently more regularly during the later phases of the infection or during the convalescence from the clinical attack than during the acute stage. The epidemiologic findings in the Louisiana outbreak (Olson and Treuting, 1944) uphold the latter.

Vervoort and Ruys (1939/40) recorded a very interesting history. In Amsterdam, an owner of many exotic birds contracted an illness which lasted for two months and was diagnosed as a very severe influenza. A few weeks after his recovery, he met a friend from Stockholm with whom he passed several hours in town, but he did not receive him at his home. The friend fell ill two weeks later in Stockholm where the disease was recognized as psittacosis. Then the blood of the two friends was sent by the Swedish physician to Professor S. P. Bedson who found the complement fixation reaction with the psittacosis antigen strongly positive. The serum of the birdowner fixed complement in a dilution of 1:128; Vervoort and Ruys therefore concluded that this is one of those "rare" instances in which psittacosis was transmitted from man to man. Why the infection of the Amsterdam patient was contagious while he conversed with his friend from Stockholm is difficult to explain, since epidemiologic inquiries failed to disclose psittacosis, severe or mild, among members of his family or among his friends.

The inductive analysis of the few facts in this unusual transmission chain suggests that the birdowner who had recovered from a severe attack of psittacosis was a convalescent shedder of the virus at least two and one-half months after the onset of his illness. It is reasonable to assume that the patient was free from suggestive symptoms when he was examined by Vervoort and Ruys about 6 months after he is supposed to have transmitted the infective agent, or otherwise, the well-known and competent Dutch investigators would have searched his sputum for the infective agent.

Haagen and Mauer's case (Haagen and Mauer, 1938) also deserves consideration. The offensive purulent sputum of a bird dealer with clinical bronchopneumonia and one large and several small lung abscesses yielded a psittacosis agent. Positive evidence of psittacosis was also found at autopsy. Since the clinical picture and the postmortem findings did not correspond to those of psittacosis, these two German workers considered the patient a carrier and possibly a shedder after a preceding abortive or latent infection.

A similar observation on another birdbreeder was made in California and was reported in part by Hoge (1934). In this case purulent bronchopneumonia followed severe typical psittacosis. Unpublished experimental studies by Meyer and Eddie led to the conclusion that a clinically active or a latent psittacosis infection of the respiratory tree, in rare instances, encourages a secondary staphylococcus bronchopneumonia. The cases reported by Haagen and Mauer and by Hoge must be interpreted in this sense.

In the case reported herein, two factors continuously suggested the possibility that the patient might be a carrier: paroxysmal coughing spells, particularly in the morning, and the level of complement fixing antibodies against the psittacosis agent, which occasionally rose to 1:256++++ throughout the 12 years.

Systematic search for virus in sputum specimens from 12 other patients between 1931 and 1936, before therapeutically effective antibiotics were available, had yielded positive findings in two instances on the 32nd day after the onset of the disease (Meyer and Eddie, 1939). Subsequent intraperitoneal injection of properly prepared sputum specimens invariably proved noninfective for mice. In the majority of the patients who recovered in this group, the agent disappeared from the respiratory tract within 35 days, while in the case of the carrier,

it was isolated from the sputum 10 years after the original infection.

In 11 cases in which patients recovered from psittacosis of varying severity and follow-up study was possible, the complement fixation antibody titer of the serum declined within 12 months to 1:8 or lower (Meyer and Eddie, 1939; Vervoort and Ruys, 1939/40). However, the level may remain fairly high for several years. For example, in one of the 11, it was 1:32 at the end of three years, and in two others, 1:16++++, 4 and 5 years, respectively, after the clinical illness. Whether these 11 persons, who contracted their infection before 1943, still harbor the psittacosis agent in their tissues is not known. In any event, two have been examined repeatedly, but since they have presented no evidence of an infective process localized in the respiratory tract and apparently, have not served as a source for further infections, it seemed unnecessary to make further studies to determine if they were carriers.

Ever since laboratory evidence fully supported the idea that our patient was a carrier, all subsequent patients encountered have been examined with the point of view that they too might remain infective shedders. In these cases, complement fixation titers of as high as 1:128++++ have fallen to 1:2 within 12 months. There is ample evidence that early treatment with moderately bactericidal antibiotics (penicillin and now aureomycin) assists the natural defense of the host and prevents the carrier stage.

From these over-all considerations, attention turns to the infecting agent. The strains of psittacosis-like agent isolated in outbreaks of high human infectivity possess the dominant characteristics of specific mutants with little relationship to each other. For example, the Louisiana (Borg) pneumonitis strain fatally infects guinea pigs. It is endowed with a highly powerful endotoxin, specifically neutralized only by the homologous antiserum. Intense pathogenicity for mammals in part explains the extreme human-to-human infectivity. On the other hand, the San Francisco strain with similar characteristics of infectiousness, when injected intraperitoneally does not infect mice; it has a low virulence for birds and less tendency to induce latent infections. It differs from the meningopneumonitis virus in toxin neutralization tests; the antiserum inactivates only the homologous toxin and that of strains MD and HP, which as laboratory infections are direct descendants. The Illinois pneumonitis agent, virulent for mice infected by the intraperitoneal route, protects the mice against intracerebral inoculation of San Francisco virus. A moderately active Illinois endotoxin is neutralized by the Mu46 antitoxin.

The carrier strain, Mu46, cannot be differentiated through pathogenicity tests from a great many parrot and parakeet strains isolated in recent years. Furthermore, the toxin and virus neutralization tests established this relationship with the exception that the specific Mu46 serum exhibited a very limited range of protection and only neutralized the virus of meningopneumonitis.

It is apparent from this account that the human pneumonitis and the carrier strain exhibit differences in no way greater than those found among the strains of a number of similar agents. One would expect the Mu46 strain, which had been in the tissues of man for 8 years to show certain dominant qualities characterizing it as a "humanized" psittacosis agent. It is by no means inconceivable that such a change in biologic peculiarities of the agent would in time occur and that, given suitable con-

ditions, "a specifically human infection spreading like low-grade smallpox" (Burnet, 1945) might arise. The Louisiana bayou outbreak and many of the recent hospital outbreaks make it seem likely that changes do occur.

Massive elimination of the infective agent probably occurs only when the patient is seriously ill, so that usually only hospital contacts become infected. However, the observations of Gerlach and of Vervoort and Ruys attest to the possibility that conditions might arise in which a carrier under the influence of fortuitous, but at the present unknown, factors would liberate effective amounts of a psittacosis-like agent.

Future epidemiologic investigations of outbreaks of human pneumonitis associated with representatives of the psittacosis-lymphogranuloma agents should include human, as well as avian, sources of infection. Information concerning the persistence of the psittacosis agent in man is desirable and can possibly be secured by repeated examination of the respiratory secretions of convalescents.

Aside from a dependable inoculation technique on suitable susceptible mice, it is essential that preferably pooled, properly preserved sputum specimens be collected daily and submitted to the laboratory. This persistent collecting of specimens coupled with the availability of antibiotics to aid in the elimination of the concomitant bacterial flora in the sputum collected from our patient led to the discovery of the carrier stage.

The bacterial flora can thus be eliminated from sputum specimens from psittacosis patients without filtration, and consequently larger amounts of the secretions can be tested. Whether this opportunity will ever be used extensively is obviously a matter of conjecture. The picture has changed in this respect since treatment with penicillin or aureomycin effectively sterilizes the respiratory discharges, and in several instances the sputum in proved cases of psittacosis was devoid of the infective agent on the 6th day after early initiation of treatment.

SUMMARY

The history of a convalescent carrier of the psittacosis agent for 10 years is detailed. A 36 year old biochemist contracted a severe psittacosis infection; the agent was demonstrated in the blood and in the sputum. After recovery, a productive cough persisted and annual re-examination of the serum invariably yielded a positive psittacosis complement fixation in titers varying between 1:128 and 1:256. In the 8 years after his initial infection, the psittacosis agent was repeatedly isolated from 24 hour specimens of sputum, before and after treatment with penicillin. Isolation was accomplished with antibiotics which inhibited in the sputum the concomitant bacterial flora highly virulent for mice. In the 10th year after his infection, the viral agent continues to be present in the respiratory secretions. Careful epidemiologic inquiries fail to indicate that the carrier was, or is, infective.

The infective agent isolated from the carrier who had been exposed to a parakeet virus behaves in pathogenicity tests like the original strain. The endotoxin is not neutralized by the antitoxin generated in roosters by the Louisiana and San Francisco pneumonitis agents. However, the carrier strain antitoxin has a broad neutralization spectrum. A protective antiserum prepared on roosters inactivates significant units of only the meningopneumonitis virus (Francis and Magill, 1938). The mouse pneumonitis (Bar Harbor), meningopneumonitis and ornithosis (pigeon 207) antiserum neutralize varying quantities of infective units of the carrier strain. Intracerebral

cross-protection tests of mice infected by the subcutaneous route in part confirm the neutralization tests. In guinea pigs, the carrier strain induces considerable resistance against the Louisiana pneumonitis agent.

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DYNAMIC ASPECTS OF THE NITROGEN METABOLISM OF *PLASMODIUM GALLINACEUM* IN VIVO AND IN VITRO

NEAL B. GROMAN*

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

The metabolism of the malarial parasite has been the subject of broadened consideration in recent years. The rapid development of the parasite and the magnitude of its growth stresses the importance of its nitrogen metabolism. The problem of how the malarial parasite converts the nitrogenous products of the host into parasite protoplasm has been considered in the present work. It has been approached by studying changes which occur in important nitrogenous fractions of chicken erythrocytes parasitized by *Plasmodium gallinaceum* under a variety of experimental conditions in vivo and in vitro. The results of these investigations reveal the degradative pattern and potential of *P. gallinaceum*, indicate the existence of a secondary relationship between the aerobic oxidative metabolism and nitrogen metabolism of the parasite, and emphasize the dynamic nature of the host-parasite relationship.

Except for interest in the destruction of hemoglobin and the accumulation of malarial pigment, studies on the nitrogen metabolism of malaria have until recently been limited. Christophers and Fulton (1938) recorded definite increase in nonprotein nitrogen (NPN), slight increase in Van Slyke amino nitrogen, and no change in ammonia upon incubation of erythrocytes parasitized by *Plasmodium knowlesi*. Moulder and Evans (1946), studying the nitrogen metabolism of *P. gallinaceum*, found that parasitized chicken erythrocytes produced large amounts of amino nitrogen in the presence of glucose as compared to normal controls, and that in the absence of glucose much of the amino nitrogen appeared as ammonia. They also found that the production of amino nitrogen was strongly inhibited by anaerobiosis, which suggested a possible linkage of the hydrolysis of protein to oxidative processes in the intact malarial parasite. Anfinsen et al. (1946), studying nitrogen requirements for in vitro culture of erythrocytes infected with *P. knowlesi*, added an amino acid mixture a priori to their culture medium, and in addition found a definite requirement of purines and pyrimidines for growth. More recently methionine has been shown to be an essential building block both in the in vivo and in vitro growth of *P. knowlesi* (McKee, Geiman, and Cobbey, 1947; McKee and Geiman, 1948).

Present evidence indicates that the malarial parasite obtains its nitrogen mainly through the breakdown of hemoglobin. Moulder and Evans (1946), studying proteinase activity of cell-free extracts of *P. gallinaceum*, found horse and chicken hemoglobin was split slowly or not at all, but found acid denatured horse or chicken globin rapidly hydrolyzed. Ball et al (1948) were unable to demonstrate hemoglobin breakdown with extracts of *P. knowlesi*.

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* Mr. and Mrs. Frank G. Logan Research Fellow, 1948–1950. Present address: Department of Microbiology, University of Washington, Seattle, Washington.

The production of malarial pigment results from the split of heme from globin by some as yet unknown mechanism, and the eventual deposition of heme as hematin within the parasite. Sinton and Ghosh, (1934a and b), after a comprehensive review of early ideas and methodology, studied the parasite pigment of *P. knowlesi* and concluded it was identical with hematin. Subsequent investigators (Devine and Fulton, 1941; Morrison and Anderson, 1942a), using increasingly critical techniques, have confirmed this identity and extended it to *P. gallinaceum* as well (Devine and Fulton, 1942; Rimington and Fulton, 1942). Evidence of differences in the extent of hemoglobin breakdown by various malaria species has been furnished by Black (1947) by means of a cyanol staining technique.

Christophers and Fulton (1938) first demonstrated quantitative decrease of hemoglobin due to parasite activity. A definite decline was recorded in oxyhemoglobin over a 9-hour incubation period by the oxygen capacity method. An acid hematin method, however, failed to show hemoglobin decline over a two- to 10-hour span. More recent studies (Ball et al, 1948; Morrison and Jeskey, 1948) have produced quantitative data on the breakdown of hemoglobin by *P. knowlesi*. It was found that 0.76% of total erythrocyte hematin was converted to pigment per 1% of the cells infected with an average pigmented form. This means that with an infection of one average pigmented form per erythrocyte, some 80% of the hemoglobin of infected cells would theoretically be destroyed. Ball et al (1948) found the total hematin (hemoglobin plus malarial pigment) stable over a 24-hour period, which indicated retention of all the pigment by the parasite. No spectrophotometric evidence of intermediates between hemoglobin and hematin was found, and hence it was suggested that hemoglobin breakdown occurred only after absorption by the parasite. In addition to in vivo studies, in vitro changes were also observed. A 28% decrease in hemoglobin concentration over an 8-hour period was recorded. Morrison and Jeskey (1948) also found that 4 to 38% of the total pigment of duck erythrocytes infected with *Plasmodium lophurae* was in the form of parasite pigment with an average of 22% for a group of 15 ducks. Relatively little nitrogen loss per unit cell resulted from parasite metabolism. In contrast, a 0.43% decrease in total nitrogen per 1.3% cells infected with an average pigmented form was observed with *P. knowlesi* (Morrison and Jeskey, 1948). With 100% of the cells infected this would represent a theoretical loss of 33% of the nitrogen.

Adequate review of the nitrogen metabolism of malarial parasites also requires consideration of those aspects of the respiratory and carbohydrate metabolism necessary for understanding the relationships between oxidative processes and changes in nitrogenous fractions. A comprehensive review of malarial parasite metabolism has been written by Moulder (1948) and should be consulted for this additional information.

MATERIALS AND METHODS

Parasitological methods

Strain 8A of *Plasmodium gallinaceum*, as designated by the Committee on the Terminology of Strains of Avian Malaria of the American Society of Parasitologists, was used throughout this study. Continuous passage from chicken to chicken was maintained by blood infection. Hampshires weighing from 1500 to 2000 g were used without regard to sex.

Parasite counts were made on thin smears stained with Giemsa. Parasite area was determined on similar smears by using the criteria of Silverman et al (1944) for parasite distribution as well as their formula for parasite area. Reticulo-

cytes were counted in thin smears stained vitally with brom cresyl blue and subsequently stained with Giemsa.

Normal and parasitized blood preparations

In general the procedures followed here are those described by Speck, Moulder and Evans (1946). Blood was drawn from the wing vein, jugular vein, or heart of the normal or infected chicken with 0.1 volume of 10% sodium citrate added to prevent coagulation. The citrated blood was then centrifuged for 5 minutes at 2000 r.p.m. in an angle head centrifuge.* The plasma was removed by aspiration and the cells resuspended in cold Krebs calcium-free phosphate saline, pH 7.4 (Krebs and Eggleston, 1940), to a volume 4 times that of the packed cells and centrifuged. Washing was repeated twice more and the washed cells were then suspended in phosphate-saline so as to give a final count of from 1 to 3×10^9 red blood cells (RBC) per cmm of suspension. Red cell counts were made on a Spencer bright line hemocytometer, and a sufficient number of cells were counted to reduce counting error to the 5% level. All final values expressed as micromoles per 10^{10} red blood cells were based on this count. Aliquots of the red cell suspension were used for the various analyses indicated below. Preparations for manometric work were made in the same manner.

Analytical methods

A tungstate filtrate was prepared from each sample, and appropriate aliquots were used in the determination of ammonia nitrogen, α-amino nitrogen, and nonprotein nitrogen. The final step in each of these methods consisted of the colorimetric determination of ammonia with Nessler's reagent (Vanselow, 1940). One cc of the reagent was added for every 10 cc of final volume. Samples were read on the Klett-Summerson photoelectric colorimeter with filter KS-42 after color had been allowed to develop for 30 minutes. Appropriate blanks and standards were run simultaneously with each determination.

Ammonia nitrogen.—Ammonia was determined by Archibald's method (1943), which gives quantitative yields of ammonia in the presence of labile ammonia yielding material. Speck's modification of the vacuum distillation apparatus (1949) was used. After the apparatus was connected to the water pump, 3 cc of saturated borate buffer, pH 10.1, was admitted, and the sample was distilled for 8 minutes into the receiving flask containing 3 cc of 0.05 *N* sulfuric acid. The ammonia in the distillate was then measured as described above.

Amino nitrogen.—Alpha-amino nitrogen was determined by the method of Sobel, Hirschman, and Besman (1945) using Speck's modification (1949) of the procedure and apparatus. The pH of the tungstate aliquot, normally a 2 cc sample, was adjusted to 2.4 by adding 0.1 cc of 1 *N* sodium hydroxide prior to addition of the prescribed amount of citrate buffer. It was necessary to lengthen the digestion period in the boiling water bath to 20 minutes, probably as a result of the larger volume used. Digestion was carried out directly in 50 cc centrifuge tubes each plugged by a stopper with a capillary tube inserted. Consistent recoveries of 95% to 105% of the α-amino nitrogen from standard solutions of alanine, glycine, and leucine resulted with this method. Ammonia formed from the amino acids by the action of ninhydrin was distilled into 3 cc of 0.05 *N* sulfuric acid and determined as previously described.

Nonprotein nitrogen.—Nonprotein nitrogen was measured in tungstate filtrates by Kjeldahl digestion. Digestion and distillation were carried out in the same 18×150 mm pyrex test tube, both the digestion block and steam distillation apparatus (Redemann, 1939) having been adapted to this size. Digestions were done in a solid aluminum cylinder (Alcoa ST 24, 6-inch bar stock) 6 inches in diameter and 8 inches high with 2 concentric circles of evenly spaced holes bored at one end. The holes measured 20 mm in diameter and 140 mm in depth, and were placed with their centers at radii of 1 and 2¼ inches from the center of the block. The inner and outer circles had 5 and 11 holes, respectively. A small hole of sufficient diameter to accomodate a 360 C thermometer was bored at dead center to a depth of 130 mm. The block was mounted over a one-fourth inch brass plate on an iron tripod, and was heated effectively and with minimal attention by a single Fisher burner. There was no appreciable temperature gradient from the center to the periphery of the block.

Samples containing from 0.02 to 10 mg of nitrogen were measured out into test tubes. To each sample 2 cc of a digestion mixture were added. This mixture consisted of 0.5 g mercuric sulfate, 1.0 g selenium dioxide, 75 cc concentrated sulfuric acid, 25 cc 85% phosphoric acid, and 125 cc of water. With the apparatus described, the following digestion schedule was found adequate as tested on a casein hydrolysate:*

* International Centrifuge No. 434. Head No. 804.

* The procedure adopted here is based in part on an unpublished method of Dr. M. E. Hanke of the Department of Biochemistry, University of Chicago.

1st hour	0–100 C
2nd hour	100–170
3rd hour	170–250
4th hour	250–320
5th hour	320–hold

After digestion the samples were cooled, diluted slightly with distilled water, and neutralized to a faint phenolphthalein end point with 5 *N* sodium hydroxide. The tube was then attached to the steam distillation apparatus (Redemann, 1939). Five cc of saturated borate buffer was admitted, and approximately 20 cc of distillate were collected in a graduated receiving tube containing 2 cc of 0.05 *N* sulfuric acid. Nessler's reagent was added, the samples diluted to a final volume of 25 cc, and read after the 30-minute interval.

Total nitrogen.—A suitable sample of the red cell suspension was made by diluting further with phosphate-saline. An aliquot was then digested and distilled in the same manner as the nonprotein nitrogen samples. Owing to the large amount of nitrogen involved, aliquots of the distillate were taken prior to nesslerization.

Blood pigments.—A quantitative colorimetric determination of soluble blood pigments and the insoluble pigments deposited during malarial infection was based on a procedure of Morrison and Anderson (1942b). In this procedure final color was developed as ferrihemic acid (acid hematin). For purposes of convenience and because of the practically quantitative identity, soluble pigment will be referred to as hemoglobin and insoluble pigment as malarial pigment. With normal blood the total pigment and hemoglobin values were found equivalent. With infected blood, total pigment was found equivalent to hemoglobin plus malarial pigment values.

Hemoglobin was determined by adding 0.4 cc of the red blood cell suspension to 4.6 cc of a 0.1% saponin solution in 0.9% sodium chloride. The blood was allowed to lyse and was mixed intermittently for a 5-minute period. The lysed suspension was then centrifuged for 5 minutes at 2000 r.p.m. The supernatant was poured off into test tubes and was used for hemoglobin analysis. The precipitate, containing malarial pigment when the cells were infected, was treated further for the recovery of hematin as noted below.

Hemoglobin was determined by adding 1 cc of the supernatant slowly, and with mixing, to approximately 8 cc of acid-acetone (2 cc of concentrated hydrochloric acid to 100 cc of acetone). When the precipitate began to settle, acid-acetone was added to bring the total volume to 10 cc. The solution was mixed, the tubes tightly stoppered, and the color of ferrihemic acid allowed to develop for a minimum of 1 hour. These solutions were then centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was poured off into colorimeter tubes which were then stoppered. Samples were read on the Klett-Summerson colorimeter using a KS-54 filter. No difficulty involving turbidity was encountered. The acid hematin color developed from chicken hemoglobin had an identical absorption spectrum with that developed from the standard hemin solution. The spectral range was studied in a Beckman Model DU Spectrophotometer from 4000 to 6700 Angström units. An acid-acetone blank and ferrihemic acid standards were included with each determination. Recrystallized hemin (Eastman Kodak Company) prepared as alkali hematin (Snell and Snell, 1937) was used as a standard and converted to ferrihemic acid with acid-acetone. Iron analysis of the hemin was made by the method of Drabkin (1941). Computations of both hemoglobin and malarial pigment values in terms of iron content were based on a hemin iron value of 8.13%.

When infected blood was analyzed, the malarial pigment was determined in the precipitate resulting from saponin lysis. The precipitate was mixed and centrifuged in 10 cc of 0.9% sodium chloride in order to remove all soluble pigment. After the 4th washing, the wash water contained no detectable pigment. As much of the supernatant as possible was removed by aspiration, and acid-acetone was added to the precipitate to a volume of approximately 4 cc. The precipitate was then stirred until coagulation was well advanced and until no visible black particles of pigment remained. Acid-acetone was then added to bring the final volume to 5 cc. The tubes were stoppered and the subsequent procedure was identical with that described for hemoglobin.

Equivalence of total pigment to either hemoglobin in the normal animal or hemoglobin and malarial pigment in the infected animal was determined by first lysing the cells in saponin as in the other determinations, and then without centrifuging adding 1 cc aliquots of the lysed suspension to acid-acetone. With this exception the treatment paralleled that indicated above. After equivalence had been indicated a sufficient number of times, the total pigment determinations were omitted.

In vitro methods

Manometric techniques.—In vitro studies of the effects of aerobiosis, anaerobiosis, inhibitors, and substrates on nitrogenous fractions were made with a Barcroft-Warburg respirometer. When aerobic conditions were maintained, 0.2 cc of 20% potassium hydroxide was placed on a filter roll in the center cup of each vessel. Anaerobic condi-

tions were obtained by placing a small piece of yellow phosphorous in the center cup and then flushing the flasks out with commercial tank nitrogen for 3 or 4 minutes. When cyanide was used as an inhibitor, the center cup contained a cyanide potassium hydroxide mixture as prescribed by Umbreit, Burris, and Stauffer (1945). All determinations were made at a temperature of 40 C. Measurements of pH were taken with a Coleman model 3 electrometer. Duplicate flasks were run in most cases where oxygen uptake was measured, and the blood from the duplicates was pooled prior to making a tungstate filtrate and pH determination.

In vitro incubation.—Approximately 5 cc of blood were drawn aseptically from the jugular vein of an infected chicken into a sterile 125 cc Erlenmeyer flask containing a pinch of heparin to prevent coagulation. A similar sample was drawn from the jugular immediately thereafter for use in initial analyses. The blood sample in the flask was flushed with a 5% carbon dioxide-95% air mixture passed through a cotton trap to prevent bacterial contamination. After a thorough mixing and flushing, both the inlet and outlet tubes to the Erlenmeyer were closed off and the flask was placed on a shaker which carried it through 25 cycles of a 5 cm oscillation per minute. The whole apparatus was placed in a 37 C incubator. At the end of the incubation period, blood agar plates were streaked to check for sterility. Only sterile samples were analyzed.

EXPERIMENTAL RESULTS

Description of in vivo changes

Initial studies were made on changes in nitrogenous fractions of infected chicken erythrocytes. Total nitrogen, NPN, α-amino nitrogen, ammonia, hemoglobin, and malarial pigment values were recorded. Selection of these fractions gave information concerning nitrogenous components of varying degrees of complexity. Total nitrogen and hemoglobin indicate both generally and specifically the condition of the gross nitrogen pool within the erythrocyte. The NPN fraction in a tungstate filtrate, while inclusive of both α-amino and ammonia nitrogen, indicates additionally alterations in amounts of other small molecules having nitrogen as a constituent. These are, for example, purines, pyrimidines, and creatinine. Alpha-amino nitrogen reflects changes in the amino acids which are essential for the synthesis of parasite protein. The importance of free ammonia in an actively growing system explains its inclusion, and because of the significance of malarial pigment quantitative information regarding its production was sought.

In order to follow the course of infection in the chicken, periodic blood smears were taken after an initial infecting dose of blood. At selected intervals blood samples were withdrawn from the chicken and analyzed for all nitrogenous fractions. Certain limits to these analyses were imposed both by theoretical argument and by practical necessity. No analyses were made on plasma. Since it is extremely likely that the metabolic activity of the parasite per se is limited to the host cell, any changes due to alteration of constituents in the red cell, losses from it, or gains from the plasma during parasite activity would be reflected in changes in the erythrocytic fractions. Alterations in the host metabolism exclusive of the erythrocytes were not involved in this study. While desirable, it was not feasible to correlate changes in the nitrogenous constituents during the course of a single asexual cycle. Approximately 3 cc of blood were required to make a complete set of analyses, and frequent bleeding during infection caused quick appearance of the immature red blood cells or reticulocytes. As will be discussed later, it was not possible to correct for the reticulocyte activity. It was decided to space the samples at least 18 to 24 hours apart early in the infection and 8 to 12 hours apart during the cycle occurring at the peak of parasitemia in order to avoid the appearance of reticulocytes. Rapidly developing parasitemias reaching high peaks were the ideal

for the type of results desired.

Table 1 is a compilation of data from both normal and infected chickens. It gives the mean values of various nitrogenous fractions and the results of a statistical analysis of their significance. The *P* value of probability was derived from analysis of the significance of the differences between normal and infected means. Both low and high parasitemias have been included in the infected and after infection. All the fractions, i.e., hemoglobin, total nitrogen, NPN and α-amino nitrogen showed some variation during the normal period. Ammonia (not graphed) appeared in 2 of 5 samples with a maximal value of 1.4 micromoles per 10^{10} red blood cells. Low values for the α-amino nitrogen were characteristic, reflecting the relative metabolic quiescence of the mature red cell. Following infection there was a

TABLE 1.—*Mean values of nitrogenous constituents in normal and infected chicken erythrocytes.*

	Micromoles nitrogen per 10^{10} RBC				Micromoles iron per 10^{10} RBC
	Total nitrogen	NPN	α-amino nitrogen	Ammonia	Hemoglobin
	Normal chickens				
Mean values	5667	70.1	11.1	0.7	24.8
Standard deviation	712	10.4	3.6	1.2	1.6
Number of samples	22	21	22	22	39
	Infected chickens				
Mean values	5631	93.8	28.9	2.7	23.1
Standard deviation	815	28.6	15.6	3.4	2.2
Number of samples	16	26	26	26	17
*P**	0.9	<1:10,000	<1:10,000	0.04	0.005

* Probability of the significance of the difference between normal and infected means.
For the normal chickens the reticulocyte counts averaged less than 2%. For infected chickens all reticulocyte counts were less than 15% with 60% of the counts under the 1% level. Parasite counts ranged from 0 to 16,800 parasites per 10^4 red blood cells with the average count 6810 parasites per 10^4 red blood cells.

samples. The differences between normal and infected samples, therefore, are not maximal, so that any significant differences which occur take on added meaning. In infected animals there is a pronounced increase in NPN and α-amino nitrogen over the normal controls. The decrease in hemoglobin as a result of infection is also significant. Though ammonia shows over-all increases, its appearance in both normal and infected chickens is erratic. Some samples are devoid of ammonia nitrogen while others contain measureable quantities. The total nitrogen of the cells remains constant.

The general conclusions derived from the mean values are reflected in an examination of individual cases. Variations from the general pattern also appear. Figure 1 represents a study of a single chicken for several days before rapid rise of the parasite count with a parallel increase in the percent cells infected. Reticulocyte counts characteristically remained low until the peak of parasitemia had been reached, and then rose precipitously. In the initial stages of infection the appearance of malarial pigment was the only evidence of change. As the parasite count rose both the NPN and α-amino nitrogen values increased. Both the hemoglobin and total nitrogen values remained stabilized at a slightly lower level, and the malarial pigment continued to accumulate. Small amounts of ammonia (not graphed) appeared but showed no relation to the development of the infection. In almost all details this is the prototype of normalcy and infection.

Two additional examples of changes occurring during infection are illustrated in figure 2. Chicken 7702 portrays a

relatively low grade infection complicated by an early reticulocyte influx. Chicken 7708 is an example of a high parasitemia characterized by its unusual duration, and in which the analyses were carried well past the period of the initial reticulocyte influx. Little effect of the parasitemia is noted in 7702. The rise in NPN and α-amino nitrogen before the appearance of parasites implicates the reticulocytes. This will be discussed in the following paragraph. In the infection of 7708, charges in NPN and α-amino nitrogen followed the basic pattern previously described. Malarial pigment accumulated, but there was a decline in hemoglobin which continued throughout the course of the infection and which was considerable in contrast to the mean decline shown in table 1. This drop was due to possibly two factors: (1) the loss in hemoglobin per unit cell due to parasite destruction, and (2) the large influx of reticulocytes which may have had a lower hemoglobin content.

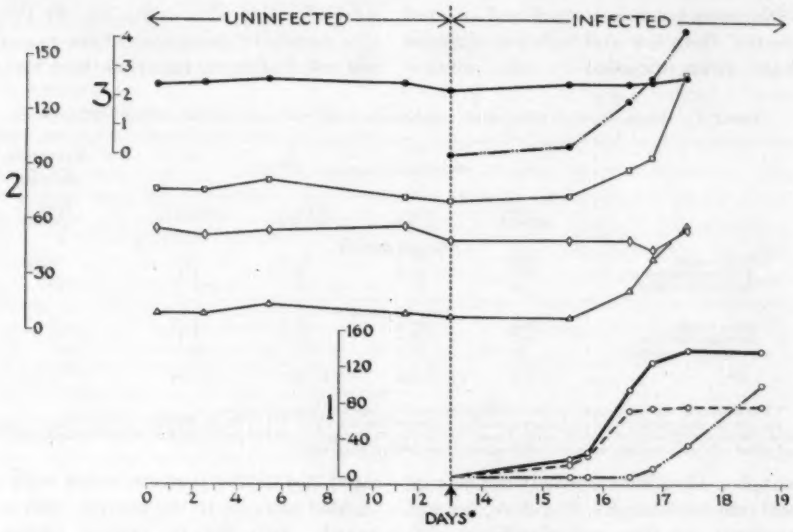

FIG. 1.—In vivo changes in nitrogenous fractions of chicken erythrocytes.

* Note the different scales used for the days of infection; black arrow, single dose of infected blood given at this time. Scale 1: solid line, 10^2 parasites per 10^4 RBC; broken line, percent cells infected; line and dots, percent reticulocytes. Scale 2: diamond, 10^2 micromoles total nitrogen, square, micromoles NPN; triangle, micromoles α-amino nitrogen (all per 10^{10} RBC). Scale 3: solid line, 10^1 micromoles iron, total pigment; line and dots, micromoles iron, malarial pigment (both per 10^{10} RBC).

It was hoped that corrections for reticulocyte influx could be made in order to divorce the effects due to their presence from those due to parasite activity. Accumulation of data on the metabolic behavior of the reticulocytes indicated this was not possible. Examination of both graphs in figure 2 reveals that the first influx of reticulocytes did not affect the hemoglobin values, but that prolonged drainage of the hematopoietic centers (7708) eventually

brought about a decline in the hemoglobin values per unit cell. Surface area measurements by Silverman et al (1944) show that chicken reticulocytes have on the average a larger volume than normal chicken erythrocytes. If the initial reticulocytes were only slightly deficient in hemoglobin their larger volume would compensate for this deficiency, and no apparent decline per unit cell would be noted. As the more immature cells appeared in the circulating blood, this volume compensation would no longer balance the greater hemoglobin deficiency and the drop would now become apparent. Any attempt to compensate for hemoglobin due to reticulocyte entry would have to account for this gradient which would vary considerably with the character of the demands on the blood forming centers.

Other facts concerning reticulocytes also emphasize the difficulty of trying to compensate for their presence. Changes in NPN and α-amino nitrogen in reticulocytes resembled those found in parasitized mature red cells. This is illustrated in figure 2 and in data gathered in table 2 (7701 and 7706), in which values are arranged in chronological sequence. There was a continued rise or maintenance of heightened NPN and α-amino nitrogen in the face of parasite decline. An uninfected chicken, 227, was bled frequently to induce reticulocyte influx, and here again increases in NPN and α-amino nitrogen were noted. In the hope of obtaining a reasonable correction factor for the reticulocytes, attempts were made to correlate reticulocyte counts or area with variations in nitrogenous fractions, but these were unsuccessful. Similar difficulty was encountered by Silverman et al (1944) in attempting to correlate oxygen uptake with reticulocyte area.

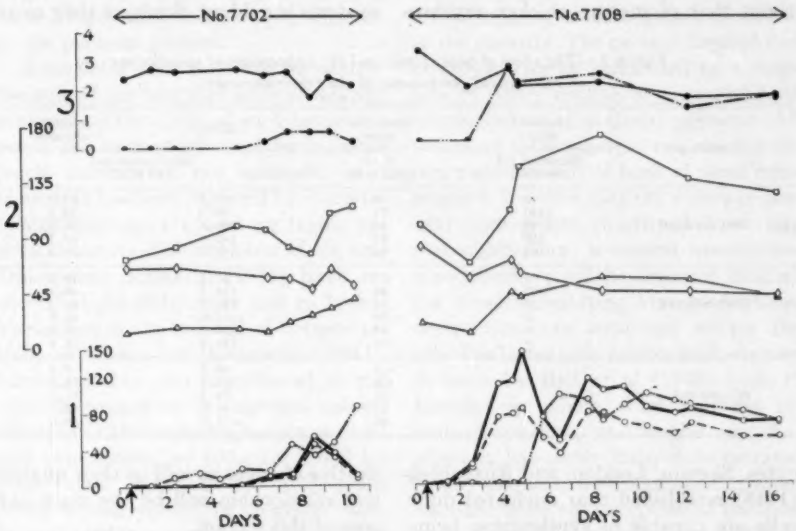

FIG. 2.—In vivo changes in nitrogenous fractions of infected chicken erythrocytes. The symbols and their meanings are identical with those given in figure 1.

The evidence gathered here shows that reticulocytes possess metabolic activity far in excess of that of the mature red cells and comparable to that of many preparations of parasitized erythrocytes. Other data support this concept. Silverman et al (1944) and Moulder (1949) found the oxygen uptake of reticulocytes approximately 5 times that of mature chicken erythrocytes. Shemin, London, and Rittenberg (1948) established that nucleated duck cells are capable of synthesizing heme in vitro. One might postulate that the reticulocytes of the chicken are similarly engaged, and reason further that the end product of this synthesis is hemoglobin. Such activity would account for the increased turnover in the nitrogen fractions as compared with mature erythrocytes, and the changes occurring upon their entry. The lack of correlation with reticulocyte counts noted above may be the result of individual differences in metabolic rates.

TABLE 2.—*The effect of parasitemia and the appearance of reticulocytes on nitrogenous components of chicken erythrocytes.*

Chicken no.	Parasite count	Reticulocytes	NPN	α-amino nitrogen
	Parasites per 10^4 RBC	% of total cells	Micromoles per 10^{10} RBC	
7701, infected	0	0	77.5	12.7
	1150	27	98.3	24.1
	1720	49	100.6	30.2
	260	84	142.3	43.7
	60	94	185.2	51.2
7706, infected	0	<1	67.1	10.5
	7060	48	116.7	40.9
	25	61	147.5	53.2
	0	56	121.0	42.9
	0	45	130.8	35.7
	0	34	133.5	31.9
227, normal		<1	60.8	9.4
		<1	63.9	15.5
		17	71.2	17.2
		17	84.4	18.5
		36	75.6	16.8

Comparison of qualitative and quantitative changes in vivo and in vitro

Comparison of the in vivo changes in parasitized chicken erythrocytes with those obtained on in vitro incubation is of twofold interest. There is a gain of insight into the dynamics of the in vivo system because of the lesser variability of the in vitro system, and in addition such information confirms the over-all reliability of the in vitro system as a valid indicator of parasite metabolism. A qualitative similarity between the two systems is evident. Study of their quantitative changes as well as their qualitative relationship will be the main purpose of this section.

Total nitrogen.—The total nitrogen pool within the erythrocyte which is available to the invading parasites averages 5667 micromoles per 10^{10} red blood cells (table 1). By suitable calculations it is found that an average of 4650 micromoles or 82% of the total nitrogen available is in the form of hemoglobin. This leaves approximately 950 micromoles of nitrogen unidentified after taking into account an average of 70 micromoles of NPN. This remaining 17% of nitrogen is probably largely nucleoprotein plus small amounts of cytoplasmic protein exclusive of hemoglobin. There has been no evidence to date that the malarial parasite utilizes nucleoprotein as a major source of nitrogen. The total

nitrogen pool remains quite stable during infection, but there is a decrease in hemoglobin nitrogen equivalent to 320 micromoles of nitrogen per 10^{10} red blood cells. A small increase in NPN of 20 micromoles accounts for only a small percentage of the hemoglobin decline. The remainder is probably present in larger nitrogen-containing molecules, such as peptides and polypeptides, or in the parasite protein.

Hemoglobin and malarial pigment.—Because of the fact that interest was not centered on the study of such factors as sex or age as they influence hemoglobin levels these were not critically controlled. It has been reported by Harmon (1936) that age, sex, and egg laying are critical factors in hemoglobin levels, and Dukes and Schwarte (1931) have recorded slight differences due to breed. Variations in the method of determination, as shown by Bankowski (1942), have probably also contributed to the wide discrepancies in reported values. Harmon (1936) reported values of 15.27 g of hemoglobin per 100 cc of blood for adult male chickens, and 9.55 g for adult, female, nonlaying chickens. Bankowski (1942) reported a mean value of 9.83 g of hemoglobin per 100 cc of blood for young White Leghorn pullets. In the present studies, the mean value obtained for 13 normal chickens was 12.2 g of hemoglobin per 100 cc of blood based on an average value of 3×10^6 red cells per cmm of blood. It was necessary to use an average count in the calculation since all analyses were based on 10^{10} red blood cells. With this in mind and in view of the lack of knowledge concerning important variables, such as sex of the chickens, this mean value is considered reasonable.

A quantitative estimate of hemoglobin destruction by *P. gallinaceum* can be made from the data gathered. Certain assumptions, however, are required to make such an estimate. There is no stable base value for calculating the percent destruction of hemoglobin as shown by the normal variation in the total pigment values (see fig. 1). As a simplifying assumption the total pigment of the cell at the time of analysis, as represented by hemoglobin plus malarial pigment, will be considered the initial amount of hemoglobin available to the parasite. The percent hemoglobin destroyed can be calculated as a function of either decline of hemoglobin or accumulation of malarial pigment. All values of total pigment rely on the direct measurement of both of these components. In order that the values of percent hemoglobin destroyed have any real significance, a second assumption is required. It will be assumed that all the hematin resulting from hemoglobin destruction has remained within the cell. The latter assumption finds support in work by Ball et al (1948) with *P. knowlesi* in which total pigment remained constant, and in the fact that pigment has never been demonstrated to any extent in the plasma of infected animals. With the exception of the data on the rate of hemoglobin destruction it was not possible to consider time as a factor. The percent destruction of hemoglobin as calculated from individual samples is necessarily given without knowledge of the length of time required for such destruction to occur. Such values mean, unless explicitly stated otherwise, that this percent destruction of hemoglobin has occurred when the particular parasite count, distribution, or combination of factors has been attained, in fact or in hypothesis, regardless of the time required for the production of such a state. It has been suggested (Lawson, 1919) that a single parasite may infect a series of cells in the course of its development. Such a sequence of events would affect esti-

mates of hemoglobin destruction. Since the parasite retains the pigment resulting from hemoglobin destruction, the values for total pigment would ultimately exceed the amount originally present. This possibility is virtually excluded by the data to be presented. It will be assumed that the parasite remains within the red cell originally infected throughout the course of its development.

The average destruction of hemoglobin can be calculated from data in table 1. The mean decline in hemoglobin, or, alternatively, the mean accumulation of pigment for the samples examined is 1.7 micromoles of iron. Based on an initial value of 24.8 micromoles, this represents a loss of 6.9% of the total hemoglobin for an average parasitemia of 6810 parasites per 10^4 red blood cells. Such calculations, however, give no idea of parasite forms involved or the limits of destruction. The destructive power of the parasite is reflected more accurately by basing calculations on the percent cells infected, and may be refined further by basing calculations on percent cells infected with pigmented forms only. It is probable, however, that ali parasite forms, with the possible exception of the segmenters, are engaged to a greater or lesser degree in hemoglobin destruction. Measurements of malarial pigment in this study are limited to those pigment particles capable of being sedimented by low speed centrifugation. One would predict then that calculations based on the percent cells infected with pigmented forms, i.e., those with microscopically visible pigment granules, would give the best available indication of the extent of hemoglobin destruction by the parasite. Any heme which had been split from the globin molecule and which was present as subsedimentable particles or in some soluble form would be determined as hemoglobin in the supernatant. Thus, the results given here, which are based on the percent cells infected with pigmented forms, probably represent somewhat less than maximum values for hemoglobin breakdown or malarial pigment recovery.

TABLE 3.—*Destruction of hemoglobin in chicken erythrocytes infected with* P. gallinaceum.

Sample no.	% cells infected with pigmented forms	Micromoles of iron per 10^{10} RBC		% hemoglobin destroyed of cells infected with pigmented forms	% total hemoglobin destroyed
		Total hemoglobin initially available	Hemoglobin destroyed*		
1	7.6	23.5	0.2	10.6	0.8
2	33.0	23.1	1.8	24.0	7.9
3	13.8	24.3	0.4	11.9	1.6
4	25.3	23.3	2.1	35.6	9.0
5	57.1	27.2	2.8	18.0	10.3
6	45.1	21.4	2.2	23.3	10.5
7	7.4	23.5	0.3	17.2	1.3
8	24.6	26.9	1.9	28.7	7.0
9	5.1	24.3	0.3	24.2	1.2
10	28.2	24.3	1.8	26.3	7.4
11	31.8	25.2	2.6	32.4	10.3
12	6.8	22.4	0.6	38.1	2.6
13	39.6	25.0	1.9	19.2	7.6
14	34.2	24.3	0.7	46.7	2.9

* These values were determined by direct measurement of malarial pigment.

Examples of hemoglobin destruction are collected in table 3. The percent hemoglobin destroyed in cells infected with pigmented forms is given, and for comparative purposes the percent of the total pigment destroyed is also included. The amount of hemoglobin destroyed is reported directly as malarial pigment deposited within the red cell. Parasitemias range from 1240 to 16,000 para-

sites per 10^4 red blood cells with an average value of 7980. The percent cells infected with pigmented forms vary from 5.1% to 57.1%, with a mean value of 26.8%. The percent hemoglobin destroyed in cells infected with pigmented forms ranges from 10.7% to 45.7%, with a mean value of 25.4%. This indicates that in an infection in which each cell would be host to a pigmented form, 25.4% of the hemoglobin which was initially available would be destroyed. This value does not, however, take into account the variability of the pigmented forms. Attempts have been made to correlate pigment production with total parasite count, percent cells infected with pigmented forms, parasite area, and combinations thereof in order to analyze this variance. The results have been unsatisfactory. The asynchronous nature of *P. gallinaceum* is at least in part responsible for the difficulty. In the in vitro work, correlations of oxygen uptake with parasite area have been obtained which agree well with the values of Silverman et al (1944). This indicates that other factors, which do not influence the rate of oxygen uptake, may be operating in the production of pigment.

Further data on hemoglobin destruction was sought in the in vitro work. In the manometric studies, which will be described later, no significant changes in the amount of hemoglobin or malarial pigment could be detected during periods up to 4½ hours in duration. In two studies (table 4) using the alternate in vitro technique, parasitized whole bood, was incubated for a period of 14 hours, and significant increases in malarial pigment were observed. The increased parasitemia in chicken 7788 and the maintenance of erythrocytic and parasitic integrity, as evidenced by microscopic study, indicated that the system was close to physiological.

TABLE 4.—*Destruction of hemoglobin by* P. gallinaceum *on in vitro incubation.*

Chicken no.	Parasites per 10^4 RBC	% cells infected	% cells infected with pigmented forms	Hemoglobin initially available	Hemoglobin destroyed*	% hemoglobin destroyed of cells infected with pigmented forms	Parasite distribution as % of parasite forms		
				Micromoles iron per 10^{10} RBC			S†	M†	L†
7787									
Initial	4700	22.9	22.9	20.2	2.04	43.0	0	52	48
Final	4500	27.1	27.1		2.55	46.5	0	55	45
7788									
Initial	7100	50.9	5.9	24.3	0.67	46.7	22	76	2
Final	8400	54.6	34.2		1.47	17.7	8	89	3

* Measured as malarial pigment.
† Small forms (S) are young parasites containing practically no cytoplasm. Medium forms (M) include small forms with a little cytoplasm, to forms with four nuclei. Large forms (L) include parasites with 5 or more nuclei and all segmenters.

With the data in table 4, probable limits of hemoglobin destruction per unit cell can be estimated. In 7787 almost 50% of the parasites were large forms, and more than 99% of the parasites were pigmented. Furthermore, 72% of the cells were multiply infected, i.e., had two or more parasites per cell. This represents a very mature parasitemia in the sense that in each cell which was infected the amount of hemoglobin destroyed was close to maximum. This conclusion is supported by the observation that only a small increase in the percent hemoglobin destroyed occurred over the 14-hour incubation period. This is significant in view of the fact that the parasite population was static and therefore no new cells had been invaded. Under these circumstances 46.5% of the hemoglobin of the infected cells was de-

stroyed. Expressed in a different way, this means that if every red cell were multiply infected at the same time a probable maximum of 46.5% of the hemoglobin available would be destroyed during the course of a single asexual cycle. The asynchronous nature of *P. gallinaceum* makes such a sequence of events highly improbable, and accounts for the much lower average percent of total pigment destroyed found in actual infections.

From the data on 7788 (table 4), it was possible to calculate the maximum destruction of hemoglobin by a single parasite within the red cell. A microscopic study of the character of the infection showed that the large forms were distributed one per red cell, and that they were all pigmented. In the initial analysis 62% of the large forms were segmenters and the other 38% had 5 or more nuclei. In terms of hemoglobin destruction this means that 62% of the large forms had probably reached the limit of destruction, and the other 38% were reasonably close to that limit. All the intermediate forms were mononucleate and none of them was pigmented. As a result, all the pigment formed up to this point could be attributed to the segmenters and the other large forms. On this basis, then, the value of 46.7% of the hemoglobin destroyed of cells infected with pigmented forms represents the accumulated destruction during the complete development of a segmenter of *P. gallinaceum* within a red cell. Because only particulate hematin was measured, and because there was undoubtedly slightly less destruction of hemoglobin by the other large forms than by the segmenters, this value must be considered as only a close approximation. The fact that the maximum destruction for a singly infected cell agrees so well with that for the multiply infected cell will be discussed later.

In addition to the estimate of destruction by the mature form of *P. gallinaceum*, a reasonable estimate of the hemoglobin destroyed by a young, pigmented, mononuclear form can also be made from the data of 7788. Since no intermediate forms were pigmented at the time of initial analysis, it can be assumed that young pigmented forms which appeared in the final analysis destroyed approximately equivalent amounts of hemoglobin. Lack of essential changes in the large forms allows the assumption that the amount of pigment destroyed by them remained unchanged. From these two assumptions it can be stated that the net increase in malarial pigment was due solely to the young pigmented forms. Microscopic examination showed, also, that there was an average of 1.2 young pigmented forms in each cell infected with them. Thus, 28.3% of the cells were infected with an average of 1.2 young pigmented forms each. It can be calculated that 6.9 micromoles of hemoglobin iron was initially available to the young parasites and that there was a net gain of 0.8 micromole of malarial pigment iron due to their activity. The average destruction of hemoglobin per unit cell per young pigmented form can then be calculated to be 9.7% of the hemoglobin initially available.

The rate of destruction of hemoglobin, in addition to the extent of destruction, would be a valuable criterion in establishing the relative destructive power of the various malaria species. Table 5 contains data pertinent to the rate of destruction of hemoglobin by *P. gallinaceum*. Since initial and final measurements were made within relatively short periods of time, an average value of the total pigment is taken as the base of calculation. From 7726 and 7727 it is evident that there is a consistent rise in rate of destruction as parasitemia increases. Changes in rate due to differ-

ences in parasite distribution are more subtle. In the first analysis of 7726, however, a lower parasite count is recorded than for 7709, but both have an equivalent rate of destruction. The distribution counts of these two samples show that 7726 has a much higher percentage of large forms, 23% as compared to a final value of 6% for 7709. Hence both the number and type of parasite seem to affect the rate. Similar counts and distributions would be required in order to allow comparisons between in vivo and in vitro rates. It may be pointed out, however, that the two studies (7787 and 7788) with parasitized whole blood by the alternate in vitro technique show rates of destruction of hemoglobin comparable to those in vivo. If the average rate of destruction is calculated, it is found to be 0.27% of the total hemoglobin per hour. On the basis of a 36-hour cycle for *P. gallinaceum* this gives a destruction of 9.7% of the total hemoglobin for an average parasitemia of 7250 parasites per 10^4 red blood cells. This compares favorably with the mean value for hemoglobin destruction given in table 1 for a parasitemia of 6810 parasites per 10^4 red blood cells.

TABLE 5.—*Rate of destruction of hemoglobin by* P. gallinaceum.

Chicken no.	Parasite count	Time interval	Total pigment	Hemoglobin destroyed*	Rate of hemoglobin destruction
	Parasites per 10^4 RBC	Hours	Micromoles per 10^{10} RBC		% per hour
7707					
Initial	4015	24	23.8	0.4	0.30
Final	9730			2.1	
7709					
Initial	3240	25	25.2	0.3	0.25
Final	13100			1.9	
7726 (1)					
Initial	1930	24	24.3	0.3	0.26
Final	9480			1.8	
7726 (2)					
Initial	9480	9	24.7	1.8	0.36
Final	12500			2.6	
7726 (3)					
Initial	12500	14	26.0	2.6	0.46
Final	13800			4.3	
7727 (1)					
Initial	830	24	23.6	0.0	0.10
Final	2510			0.6	
7727 (2)					
Initial	2510	23	23.7	0.6	0.28
Final	11200			1.9	
7787					
Initial	4700	14	20.2	2.0	0.24
Final	4500			2.6	
7788					
Initial	7100	14	24.3	0.7	0.21
Final	8400			1.5	

* Determined as malarial pigment.

Nonprotein and α-amino nitrogen.—Increases in NPN and α-amino nitrogen have been observed during in vivo growth of the parasite. In vitro experiments also reflect these same qualitative increases even though carried out over relatively short periods of time. Table 6 shows changes in the NPN and α-amino nitrogen fractions in both in vivo and in vitro experiments. The first 5 in vitro experiments were performed in the Warburg respirometer on washed parasitized blood. The incubation period was 4 hours and glucose was used as substrate. The 6th experiment involved

TABLE 6.—*Changes in NPN and α-amino nitrogen in chicken erythrocytes infected with* P. gallinaceum.

In vivo				In vitro			
Chicken no.	Final parasite count	Change in NPN	Change in α-amino nitrogen	Chicken no.	Final parasite count	Change in NPN	Change in α-amino nitrogen
	Parasites per 10^4 RBC	Micromoles nitrogen per 10^{10} RBC			Parasites per 10^4 RBC	Micromoles nitrogen per 10^{10} RBC	
7707	16000	21.1	9.0	7710	9380	38.7	36.8
7708	14450	47.8	20.7	7717	14300	38.2	32.0
7709	13100	5.5	9.9	7718	14300	43.2	36.4
7726	12500	21.0	31.8	7725	15650	39.1	32.4
7727	11200	17.8	30.5	7726	16800	46.7	34.8
				7788	8400	126.7	75.4

incubation of parasitized whole blood for 14 hours by the alternate in vitro technique.

The mean increases in NPN and α-amino nitrogen in vivo are given in table 1 as 23.7 and 17.8 micromoles, respectively. This seeming equivalence of change is not confirmed on examination of the individual samples in table 6. Larger increases of NPN as compared to α-amino nitrogen increases are recorded, and the converse is also true. In the manometric experiments there is a consistent production of only slightly greater amounts of NPN than α-amino nitrogen. These essentially quantitative differences can be explained by the differences in the systems they reflect.

Ammonia.—As has already been pointed out, appearance of ammonia in vivo is erratic, and when it appears the quantities are small. The appearance of ammonia in in vitro studies is related to the conditions of the experiment. Elaboration of this point will be made in the second section of this study. For the present it will suffice to say that under certain conditions large quantities of ammonia have appeared in manometric studies, and sizable increases were noted when the alternate in vitro technique was employed.

The relationship between oxidative processes and the hydrolysis of protein by P. gallinaceum

A relationship between hydrolysis of protein and oxidative processes in malarial parasites was first suggested by Moulder and Evans (1946). An experimental review of their work was

TABLE 7.—*Mean changes in normal and infected chicken erythrocytes on in vitro incubation.*

Conditions of the experiment	Average oxygen uptake	Change in α-amino nitrogen	Change in ammonia nitrogen	Number of chickens	Average parasitemia
	μl per 10^{10} sq. microns parasite area per hour	Micromoles per 10^{10} RBC per hour			Parasites per 10^6 RBC
Normal Glucose as substrate	0.88±0.07*	−0.47±0.70	−0.09±0.39	3	
Infected Glucose as substrate	50.8±17.3	9.6 ±3.0	0.18±0.70	11	15900
Infected No substrate	14.0±4.9	4.3 ±3.5	4.5 ±2.2	5	16560
Infected Glucose as substrate, anaerobiosis		3.0 ±2.6	0.4 ±1.3	5	13820

* Mean ± standard deviation.

undertaken, and the results obtained are summarized in table 7. This series of short term experiments supplied corroboration for the following facts: (1) normal chicken erythrocytes formed little or no amino nitrogen, (2) chicken erythrocytes parasitized by *P. gallinaceum* produced large amounts of amino nitrogen when incubated in air in the presence of glucose, (3) when glucose was absent from the medium much of the amino nitrogen appeared as ammonia, and (4) the production of amino nitrogen by parasitized erythrocytes was strongly inhibited by anaerobiosis. In order to secure a better understanding of the metabolic systems involved in these activities further studies were carried out.

TABLE 8.—*The effect of varying glucose concentrations on the production of amino acid and ammonia nitrogen.*

Exp. no.	Glucose concentration, molarity	Average oxygen uptake	Change in amino nitrogen	Change in ammonia nitrogen	Change in amino plus ammonia nitrogen
		μl per 10^{10} sq. microns parasite area per hour	Micromoles per 10^{10} RBC		
M-4	0.005	51.2	28.7	5.1	33.8
	0.01	48.3	32.0	2.9	34.9
	0.03	50.6	28.2	0.3	28.5
M-6	0.01	46.2	36.4	0.7	37.1
	0.02	46.1	44.4	−1.2	43.2
	0.03	46.7	29.2	−2.1	27.1

Two cc. of parasitized erythrocyte suspension were placed in each flask. Two-tenths cc. of the appropriate concentration of glucose was tipped in from the side arm at the start of the experimental period giving the final concentration as noted. Total time of each run was 4 hours. The gas phase was air, and the temperature 40 C.

In previous in vitro work (Moulder and Evans, 1946), glucose had been used as substrate only at a concentration of 0.01 *M*. It was necessary to determine whether this concentration of substrate was critical to the production of amino nitrogen, or whether similar effects were obtained over a range of substrate concentrations. The results of two experiments using varying glucose concentrations are given in table 8. In general the oxygen uptake of the parasitized erythrocytes remained unaffected over a 6-fold range of glucose concentrations. There was a consistent production of amino nitrogen at all concentrations, but the data suggest that a glucose concentration of 0.03 *M* may be slightly inhibitory to the production of amino nitrogen. It was concluded from these experiments, however, that the results obtained with a concentration of 0.01 *M* glucose were general, and that this concentration of substrate was adequate for future experiments. It should be noted that in all tables the oxygen uptake is expressed in terms of microliters of oxygen per hour per 10^{10} square microns of parasite area. The values for oxygen uptake agree well with similar figures given by Silverman et al (1944) for comparable parasitemias and indicate the reliability of the system used in these studies.

Certain assumptions concerning the relationship between amino nitrogen and ammonia nitrogen are necessary for interpretation of all data concerned with amino nitrogen production. In arriving at the conclusion of an inhibitory action due to 0.03 *M* glucose it was assumed that the net production of amino nitrogen was the sum of both amino and ammonia nitrogen increases. Evidence for this assumption was supplied by Moulder and Evans (1946), and receives additional support from some

work to be discussed in subsequent paragraphs. When a decrease in ammonia nitrogen occurred, it was not possible to decide whether this nitrogen had been converted into amino nitrogen or whether it was in some other form, and the net change in amino nitrogen was in question. Since a decrease in ammonia content occurred only when oxidative conditions had been improved as in the case of fumarate relief of malonate inhibition, it was assumed that a decline in ammonia nitrogen meant an equivalent increase in amino nitrogen. These assumptions were held valid in all discussions of the experimental work which follows.

TABLE 9.—*Comparison of glucose and pyruvate in the production of amino acid and ammonia nitrogen.*

Exp. no.	Substrate	Average oxygen uptake	Change in amino acid nitrogen	Change in ammonia nitrogen	Change in amino plus ammonia nitrogen
		μl per 10^{10} square microns parasite area per hour	Micromoles of nitrogen per 10^{10} RBC		
M-6	Glucose	48.5	37.5	5.1	42.6
	Pyruvate	55.8	24.0	6.6	30.6
M-7	Glucose	77.8	32.4	−1.7	30.7
	Pyruvate	65.9	31.8	12.1	43.9
M-8	Glucose	42.6	34.8	−2.2	32.6
	Pyruvate	44.4	29.7	5.6	35.3

Two cc. of washed, parasitized erythrocyte suspension were placed in each flask. Two-tenths cc. of the appropriate concentration of glucose or pyruvate was tipped in from the side arm at the start of the experimental period giving a final concentration of 0.01 *M* glucose and 0.02 *M* pyruvate. The experimental period was 3 hours for M-7 and 4 hours for M-6 and M-8. The gas phase was air, and the temperature 40 C.

The production of amino nitrogen in the presence of either glucose or pyruvate was compared. Both of these substrates were used with the same preparation of parasitized erythrocytes in order that any special significance of the glycolytic phase of carbohydrate breakdown might become apparent. This information would result since the degradation of glucose by *P. gallinaceum* is initiated anaerobically via the scheme of phosphorylating glycolysis (Speck and Evans, 1945; Marshall, 1948) and is followed by aerobic oxidation over the Krebs cycle (Speck et al, 1946), while the degradation of pyruvate occurs only aerobically over the Krebs cycle as shown by these same authors. The results given in table 9 indicate that no special significance should be attached to glycolysis. The changes were similar with both substrates. The production of ammonia when pyruvate was utilized is part of a more general problem which will be taken up later. It can be pointed out here that in all instances the initial rate of oxygen uptake with pyruvate exceeded that with glucose, but the rate declined during the course of the experiment. This was probably due to rapid exhaustion of the substrate. With the exception of experiment M-6 no decline in oxygen uptake was ever registered during an experimental period when glucose was being used as substrate.

Changes in amino and ammonia nitrogen in the absence of substrate had been previously recorded only at the end of a 4-hour incubation period in the Warburg apparatus. In checking these processes after 2 as well as after 4 hours, it was found that ammonia was produced throughout the resulting period of decline in oxygen uptake (table 10). Experiment M-6, in particular, em-

phasizes the shift to ammonia production since over the 4-hour period there was actually a net decline in the amount of amino nitrogen present. This is supporting evidence for the hypothesis of Moulder and Evans (1946) that the ammonia nitrogen results from the deamination of amino acids. These investigators found that the amount of amino and ammonia nitrogen produced was consistently present but quite variable in extent (see table 11). Smaller quantities of ammonia appeared during inhibition of a preparation of parasitized erythrocytes by malonate. In this case maximum inhibition was gradually approached over the period of approximately an hour after an initial, sharp curtailment of oxygen uptake. When fumarate was added simultaneously

TABLE 10.—*Changes in amino acid and ammonia nitrogen in the absence of glucose.*

Exp., no.	Time in hours	Glucose, 0.01 *M*	Average oxygen uptake	Change in amino acid nitrogen	Change in ammonia nitrogen	Change in amino plus ammonia nitrogen
			μl per 10^{10} square microns parasite area per hour	Micromoles of nitrogen per 10^{10} RBC		
M-5	2	−	16.2	8.7	6.5	15.2
		+				
	4	−	8.7	16.0	11.6	27.6
		+	38.1	36.4	0.7	37.1
M-6	2	−	18.7	8.2	4.6	12.8
		+				
	4	−	9.9	−5.8	22.6	16.8
		+	48.6	37.5	5.1	42.6

Two cc of washed, parasitized erythrocyte suspension were placed in each flask. Two-tenths cc of the appropriate concentration of glucose was tipped into the flasks requiring substrate giving the final concentration as noted. The gas phase was air, and the temperature 40 C.

in the absence of glucose was practically equivalent to that in the samples incubated with glucose, but this equivalence was not consistently found in the present experiments.

The production of ammonia by *P. gallinaceum*, while appearing to have no significance in vivo, becomes of importance in the in vitro analyses because of its close relationship to amino nitrogen. In general little ammonia was produced when glucose was used as a substrate. In the absence of any substrate, considerable quantities of ammonia were produced, apparently from the deamination of amino acids. Under anaerobic conditions little or no ammonia was formed, but inhibition by cyanide, which resulted in almost complete cessation of oxygen uptake, appeared to stimulate the conversion of amino to ammonia nitrogen. This effect with malonate, maximum recovery of oxygen uptake was immediate, and under these conditions practically no ammonia accumulated. When pyruvate was used as a substrate more ammonia appeared than in samples concurrently incubated with glucose. In all cases, however, oxygen uptake was declining, in all probability because of the exhaustion of pyruvate. As was pointed out previously, in the single case where oxygen uptake declined in the presence of glucose, the amount of ammonia which accumulated was equal to that found in the aliquot incubated with pyruvate.

The facts cited above seem to indicate that in a situation in which the rate of oxygen uptake is declining, there is an increase in ammonia production regardless of the initial level of that uptake. The lack of production of am-

monia anaerobically suggests even more strongly that it is a gradual decline in oxygen uptake which is of importance, rather than the abrupt cessation which occurs when anaerobic conditions are imposed. The nature of this phenomenon suggests that ammonia production results from the adjustment of certain metabolic equilibria involved in the aerobic oxidation of carbohydrate substrates. The effect of cyanide on the production of ammonia is the single fact which appears to stand unexplained by this general hypothesis. As in anaerobiosis, inhibition by cyanide also causes an abrupt cessation of oxygen uptake, but in this case there is production of ammonia. The pH changes occurring during anaerobiosis and cyanide inhibition are of the same order of magnitude so that differences in optimal pH for enzyme activity cannot be involved. On the basis of the available evidence it is necessary to postulate some function for cyanide which causes deamination to occur.

The curtailment of amino acid production in parasitized erythrocytes by anaerobiosis was observed by Moulder and Evans (1946), although parasite extracts hydrolyzed denatured globin anaerobically at an undiminished rate. On the basis of these observations, it was suggested that the hydrolysis of protein was in some way linked to oxidative processes. This latter term was used in a restricted sense limiting oxidative processes to aerobic reactions. Further information concerning this hypothesis was sought by the use of enzyme inhibitors. Cyanide and malonate were selected as inhibitors. Their selection was governed by the knowledge that in *P. gallinaceum* oxidation of substrate probably occurs by the transfer of hydrogen and electrons from the substrate through a series of respiratory enzymes (dehydrogenases, flavo-proteins, and cytochromes) to oxygen (Moulder, 1948). Malonate, specific in its inhibition of succinic dehydrogenase, curtails oxidation by slowing down the

TABLE 11.—*The effects of inhibitors and relief substances on the production of amino acid and ammonia nitrogen.*

Exp. no.	Substrates and inhibitors added	% oxygen uptake compared to glucose	Micromoles of nitrogen per 10^{10} RBC		
			Change in amino acid nitrogen	Change in ammonia nitrogen	Change in amino plus ammonia nitrogen
M-6	Glucose	100	37.5	5.1	42.6
	Glucose +CN	4	10.9	4.8	15.7
	Glucose +Malonate	28	17.5	8.0	25.5
M-7	Glucose	100	32.4	−1.7	30.7
	Glucose +CN	3	26.8	8.9	35.7
	Glucose +CN +ATP	3	25.1	16.3	41.4
	Glucose	100	32.4	−1.7	30.7
	Glucose +malonate	13	17.0	5.4	22.4
	Glucose +malonate +fumarate	56	22.5	0.8	23.3
M-8	Glucose	100	34.8	−2.2	32.6
	Glucose +CN	2	25.5	6.3	31.8
	Glucose +CN +ATP	3	16.5	23.1	33.6
	Glucose	100	34.8	−2.2	32.6
	Glucose +malonate	27	21.3	2.2	23.5
	Glucose +malonate +fumarate	68	32.0	−4.6	27.4
M-10	Glucose	100	46.2	−4.0	42.2
	Glucose +CN	2	21.2	2.6	23.8

All flasks contained 2 cc of washed, parasitized erythrocyte suspension. Final concentration of the various components were as follows: glucose 0.01 *M*, cyanide 0.001 *M*, ATP 0.005 *M*, malonate 0.02 *M*, and fumarate 0.01 *M*. Glucose was present during the equilibration period, and at the start of the experimental period the other components were tipped in. Total time of each experiment was 4 hours. The gas phase was air, and the temperature 40 C.

rate of carbohydrate utilization in the Krebs cycle, of which succinic dehydrogenase is an essential component. Cyanide by its inhibition of cytochrome oxidase prevents the activation of oxygen necessary for its combination with electrons from the substrate. Hence, one inhibits the rate of oxidation at the point of dehydrogenation of substrate and the other acts at the point of oxygen activation. The selection of these two inhibitors was based on the fact that they block oxidative pathways at a point of origin and at the terminal point.

A group of experiments in which both cyanide and malonate were used as inhibitors have been collected in table 11. It should be noted that the oxygen uptake in the presence of glucose is taken as the 100% base. Of the 4 experiments with cyanide as the oxidative inhibitor, inhibition of amino nitrogen production occurred in only 2, although in all 4 experiments oxygen uptake had been reduced over 95%. In 2 experiments testing malonate, M-7 and M-8, inhibition of amino nitrogen production was definite and the residual oxygen uptake was 13% and 27%, respectively. These experiments showed decisively that the production of amino nitrogen was not a direct function of the magnitude of the oxygen uptake.

The inhibition of amino acid production by anaerobiosis was found by Moulder and Evans (1946) and corroborated in this investigation. It was apparent from the data, however, that some production of amino acids had occurred anaerobically. On the basis of this evidence and the fact that parasite extracts could hydrolyze protein at an undiminished rate anaerobically, it was postulated that oxygen uptake as such was not the critical factor in protein hydrolysis. As was shown above, this postulate received further experimental confirmation. It was felt that some factor common to both aerobic and anaerobic processes but in greater abundance during aerobiosis was implicated. Energy seemed to fit the criteria set up since it is known that per mole of substrate the greater completeness of aerobic oxidations results in the production of more useful energy than from anaerobic oxidations. In both aerobic and anaerobic oxidations a part of the energy released may be used in the production of high energy phosphate bonds as found in adenosine triphosphate (ATP) (Lipmann and Kaplan, 1949). This stored energy is then available for a variety of metabolic activities. Investigation of the effects of some energy yielding systems on cyanide and malonate inhibition was undertaken concurrently with the initial inhibition experiments. Adenosine triphosphate was added to the cyanide inhibited system to give it a direct source of energy. Fumarate was added to the malonate inhibited system since it was considered that the partial restoration of aerobic oxidations was equivalent to increased energy. Both ATP and fumarate were added at the same time as the inhibitors in order to allow direct comparison with samples containing only inhibitor.

Experiments M-7 and M-8 in table 11 contain the results of two experiments in which ATP was used. In both instances where cyanide inhibition was countered with ATP there was no curtailment of amino acid production in the presence of cyanide alone. As a result the effects of ATP on the inhibition of amino acid production could not be evaluated. The only effect which appeared to have resulted from the addition of ATP was an increase in the production of ammonia. It was necessary to investigate whether this ammonia had resulted from the degradation of ATP rather than from the deamination of amino acids. It was decided

to set up control flasks in which increasing concentrations of adenine were substituted for ATP. A system including cyanide and glucose in addition to adenine was used since the ammonia increase with ATP had appeared accentuated under these conditions. It was found that with increasing concentrations of adenine there was increased ammonia production. Equivalent concentrations of ATP and adenine produced practically the same amount of ammonia. Since it seems hardly possible that all the adenine could have been converted into ATP before causing a rise in ammonia, at least a portion of the increased ammonia probably came from the degradation of ATP. One cannot rule out the possibility, however, that ATP as an energy source might have influenced the production of amino acids. It was not possible, however, to interpret any data in which ATP was involved because there was no way of determining how much ammonia resulted from increased activity due to its action, and how much from its degradation.

The relief of malonate inhibition by the addition of fumarate caused no significant increase in the amount of amino nitrogen produced (M-7 and M-8 table 11) even though oxygen uptake recovered 40% over the inhibition level. The major effect due to fumarate seemed to be an ability to hold down the amount of ammonia nitrogen which accumulated or decrease the amount already there.

The attempts which were made to implicate energy in the hydrolysis of protein were not conclusive. The results with fumarate tend to indicate there is no relationship, but this point will be referred to in the discussion. The more promising approach with ATP requires, first, that a careful analysis be made of the fate of the adenine moiety during incubation and, in particular, the extent of its breakdown.

DISCUSSION

An outstanding characteristic of the nitrogen metabolism of *P. gallinaceum* is that the degradative capacity of the parasite is relatively small compared to that of the monkey parasite *P. knowlesi*. The stability of the total nitrogen in erythrocytes infected with *P. gallinaceum* sharply contrasts the theoretical loss of 33% of the cellular nitrogen in a *P. knowlesi* infection in which an average pigmented form was present in each cell (Morrison and Jeskey, 1948). It is much more analagous to the small 3.5% losses in the total nitrogen of duck erythrocytes infected with *P. lophurae* as reported in the same paper. Ball et al (1948) have reported a single infection of a monkey by *P. knowlesi* in which 80% of the hemoglobin theoretically available to the parasite had been destroyed in vivo. The reliability of this figure as a general indicator of *P. knowlesi* potentialities was confirmed by Morrison and Jeskey (1948), who found that 76% of the total hemoglobin would be destroyed in a theoretical infection by *P. knowlesi* in which an average pigmented parasite was present in each erythrocyte. Calculation of a similar statistic for *P. gallinaceum* from the data presented in table 3 gives a value of 27% of the total hemoglobin destroyed if each cell were infected with an average pigmented form of *P. gallinaceum*. The validity of this figure for the percent destruction by an average pigmented form of *P. gallinaceum* can be checked by a second calculation. The theoretical values of the percent hemoglobin destroyed by the young pigmented forms and mature forms of *P. gallinaceum* have been calculated as 9.7% and 46.7%, respectively. On this basis an average pigmented form inter-

mediate between these two extremes would destroy 28% of the total hemoglobin if each erythrocyte were so infected. This is in close agreement with the previous value of 27%. Therefore, on the basis of destruction of hemoglobin by average pigmented forms of each parasite, *P. gallinaceum* is only about one-third as destructive as *P. knowlesi*.

Having compared theoretical infections of *P. gallinaceum* and *P. knowlesi*, it is of interest to see what occurs in actual infections. Ball et al (1948) have cited a single instance in which 17% of the total hemoglobin was destroyed when 25% of the erythrocytes were infected with *P. knowlesi*. In a series of infections with *P. gallinaceum* (table 3) an average destruction of only 7.5% of the total hemoglobin occurred with an average of 49% of the cells infected. Reduction of this figure for *P. gallinaceum* to a 25% infection gives a value of 3.8% as compared to the 17% for *P. knowlesi*. These values, while they do not consider differences in parasite distributions, indicate that *P. gallinaceum* destroys only a little more than one-fourth as much hemoglobin as *P. knowlesi* in an actual infection. This decided contrast in destructive potential is supported by the observation that the amount of total hemoglobin destroyed by *P. gallinaceum* rarely exceeds the 10% level even with parasite counts approaching 16,000 per 10^4 red blood cells in which 90% of the cells are infected.

A comparison of the rate of destruction of hemoglobin by *P. gallinaceum* and *P. knowlesi* also serves to emphasize the differences in the destructive potential of these two parasites. The asexual cycle of *P. gallinaceum* is approximately 36 hours long while that of *P. knowlesi* is about 24 hours in duration. The figure of 17% destruction of total hemoglobin by *P. knowlesi* given above covered a period of 25 hours. The rate of destruction of total hemoglobin based on these figures is 0.68% of the total hemoglobin per hour for a 25% infection which included all forms of the parasite. The average destruction of hemoglobin by *P. gallinaceum* is 0.27% of the total hemoglobin per hour for a 49% infection with all forms present. While this is not an absolute comparison in terms of parasite forms involved, it indicates that *P. gallinaceum*, if compared on a percent cell infected basis, destroys hemoglobin at a rate approximately one-fifth that of *P. knowlesi*.

The question arises that, if with an average parasitemia of 6810 parasites per 10^4 red blood cells a mean destruction of 6.9% of the total hemoglobin will occur, why destruction by a parasitemia of twice that intensity will rarely exceed 10%? In a sense this same question arose when the maximum destruction of hemoglobin calculated for cells which were multiply infected was approximately equal to the maximum destruction calculated for a single mature form of the parasite. It would seem from these observations that in a cell which is multiply infected, the net destruction by the parasites is not a summation of their individual potentialities but is considerably less. This observation might explain why attempts to correlate pigment production, amino nitrogen production, etc. with parasite areas and counts were not successful. By analogy one can reason that since parasite area and oxygen uptake are correlated that the respiratory activities of parasites in a multiply infected cell are independent.

From observations of the quantitative differences in changes in NPN and α-amino nitrogen of the in vivo and in vitro systems, it can be concluded that there is an exchange of products from the parasite to the surrounding environ-

ment. Analyses of the in vivo system involved changes in the erythrocytes only. Analyses of the in vitro system, in addition to changes in erythrocytes, included changes in the external environment during the experimental period. During in vivo incubation it was noted that changes in NPN were either larger or smaller than changes in amino nitrogen. In order for the change in NPN to have been smaller than that in amino nitrogen, which is a constituent of NPN, some loss of other nitrogenous constituents must have occurred. It was also noted that during in vitro incubation of the parasitized erythrocytes the increase in NPN almost without exception was slightly greater than the amino nitrogen increase. This probably indicates what would have occurred if no exchange with the external environment had taken place in vivo. That amino acids of the erythrocyte are probably released from the cells is suggested by the fact that over a 4-hour in vitro period there is a rapid accumulation of amino nitrogen. Levels of amino nitrogen are reached in this short time which are rare or have never been found during in vivo analyses even at the peak of infection. It is reasonable to assume that at least an equivalent production of α-amino nitrogen is occurring in vivo. Even discounting the greater rate of synthesis which may occur in vivo, it is apparent that losses of amino nitrogen to external environment probably occur. In addition to this exchange from parasitized erythrocytes to external environment, there is evidence that host factors flow into the parasitized erythrocyte. Many studies of the effect of the host's immediate nutritional state on parasitemias give evidence for this conclusion. The specific studies on the role of methionine in the growth of *P. knowlesi* (McKee, Geiman, and Cobbey, 1947; McKee and Geiman, 1948) suggest an uptake of amino acids from the external environment to parasite in at least one instance. The fact that total nitrogen of the infected erythrocyte is fairly constant in *P. gallinaceum* in the face of losses to the external environment indicates that there is essentially a balance between the outgoing and incoming fractions.

The relation of protein hydrolysis to the oxidative metabolism of *P. gallinaceum* has proved to be a problem of considerable complexity. The initial study by Moulder and Evans (1946) on this problem indicated that in the intact parasite there is some direct relationship between these two phases of parasite metabolism. In their use of the term oxidative metabolism, these authors used it to designate aerobic processes only. Much of the evidence they gathered, in addition to that found in the present investigation, argues against any primary relationship between aerobic oxidative processes and protein hydrolysis. The continued production of amino nitrogen anaerobically even though at an inhibited rate, the production of amino nitrogen despite almost 100% inhibition of oxygen uptake by cyanide, and the continuing production of amino and ammonia nitrogen in a system in which the lack of oxidizable substrate causes a decline in oxygen uptake are all arguments against such a relationship. On the other hand, the sensitivity of amino acid production to malonate inhibition is the single fact which would support a direct relationship between the two processes. The weight of the evidence indicates that curtailment of oxygen uptake is in itself insufficient to invariably cause cessation of protein hydrolysis. The evidence does appear to favor the thesis, however, that aerobic oxidative processes do in some manner facilitate the greater part of protein hydrolysis and

amino acid production. This does not exclude the possibility that anaerobic oxidative processes also contribute to this facilitation. An attempt has been made to implicate metabolically available energy as functioning in this system since while energy is liberated both anaerobically and aerobically, the major portion comes from aerobic processes. The most promising approach, that of using ATP as a direct source of energy, requires some other method of evaluating its effects. The breakdown of the adenine moiety of ATP, a fact of interest in itself, results in increased ammonia nitrogen and makes evaluation of the extent of protein hydrolysis impossible. It is felt, however, that this avenue of approach should not be closed. The inability of fumarate to cause any increase in amino nitrogen production in malonate inhibited parasites, even though oxygen uptake was increased, does not support the thesis of an energy function. Nevertheless, in this system succinic dehydrogenase was still inhibited, and its importance in energy production may be of significance.

In the experimental systems used in these studies there was no possible way in which to evaluate as separate entities the degradative processes from the synthetic ones. All measurements were merely samplings of the pool of low molecular weight nitrogenous compounds resulting from the net difference between these two processes. It is not inconceivable that the effects of oxidative processes on protein hydrolysis result, not from their direct activity in the degradative processes, but by their influence on the synthetic ones. Regardless of the nature of the relationship, the present evidence excludes the possibility of a direct relationship and indicates the existence of a secondary relationship between aerobic oxidative processes and protein hydrolysis.

CONCLUSIONS AND SUMMARY

1. A colorimetric method was worked out for the direct determination of malarial pigment as well as hemoglobin.

2. The changes in certain nitrogenous constituents of chicken erythrocytes infected with *Plasmodium gallinaceum* are described. Characteristic increases in nonprotein nitrogen (NPN) and amino nitrogen, accumulation of malarial pigment, and concurrent decreases in hemoglobin were noted. No significant changes were observed in total nitrogen. A statistical treatment of the data supports the validity of these conclusions.

3. Quantitative studies on the destruction of hemoglobin by *P. gallinaceum* reveal: (1) a theoretical maximum destruction of 46% of the total hemoglobin during an infection, (2) a mean destruction of 7% of the total hemoglobin in actual infections examined, and (3) an over-all rate of destruction of 0.27% of the total hemoglobin per hour for an average parasitemia.

4. A comparison of the extent and rate of hemoglobin destruction by *P. gallinaceum* in the chicken and *Plasmodium knowlesi* in the monkey led to the conclusion that *P. gallinaceum* is approximately one-fourth to one-fifth as destructive as *P. knowlesi*.

5. It was found, on the basis of calculations, that in cells multiply infected with *P. gallinaceum* the extent of hemoglobin destruction per unit cell was less than expected when the destructive potential of each parasite was considered independent of the other. A sharing of degradative products is postulated.

6. Comparison of in vivo and in vitro data showed a qualitative similarity in changes in NPN, amino nitrogen, and quantitative similarity in the rate of pigment accumulation. Quantitative differences in NPN and amino nitrogen are discussed in terms of the dynamics of the in vivo system. The existence of

an active interchange of nitrogenous constituents between parasitized cells and the external host environment is postulated on the basis of this evidence.

7. The relationship of protein hydrolysis to the oxidative metabolism of *P. gallinaceum* was investigated further. On the basis of cyanide and malonate inhibition studies and studies on the relationship between oxygen uptake and presence of substrate as related to amino acid production, it is concluded that a secondary rather than a direct relationship exists between aerobic oxidative processes and protein hydrolysis. The possibility of anaerobic oxidations contributing to the phenomenon was not excluded.

8. The production of ammonia during in vitro incubation of parasitized erythrocytes was observed to occur when the rate of oxygen uptake declined, regardless of the initial level of that uptake. Very little ammonia was produced in vivo although statistically significant increases occurred during infection.

9. Influx of reticulocytes during infection necessitated an examination of their nitrogenous constituents, and it was found that their NPN and amino nitrogen levels resembled those found in infected mature erythrocytes. It is concluded that the reticulocytes are as metabolically active as many preparations of parasitized erythrocytes.

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THE USE OF GASTRIC MUCIN TO LOWER RESISTANCE OF LABORATORY ANIMALS TO SYSTEMIC FUNGUS INFECTIONS

RICHARD E. STRAUSS AND ALBERT M. KLIGMAN*

From the Department of Dermatology and Syphilology, University of Pennsylvania Medical School, Philadelphia 4, Pennsylvania

Study of the deep fungus infections has been limited by the difficulty in consistently producing widespread or fatal infections in experimental animals. The lesions which result are frequently transient. Fatal infections can usually be produced only with massive doses of the organisms. Standardization of experimental disease and assay of chemotherapeutic agents on these infections is, therefore, difficult. Similarly difficult are studies dealing with the immunology of these organisms.

In 1932, Nungester, Wolf and Jourdonais reported that the time necessary to produce a fatal infection in mice with various bacteria was shortened when the organisms were suspended in hog gastric mucin. Subsequently, other investigators (Fothergill et al, 1937; Griffiths, 1942; Miller, 1933; Mills and Colwell, 1939; Nungester and Jourdonais, 1936) showed that host resistance was markedly lowered by gastric mucin, and fatal infections could be produced with organisms which ordinarily did not kill laboratory animals except with massive doses. There are many hypotheses as to how this effect is produced. It is thought that the mucin coats the organisms and prevents their destruction by the body defenses of the host (Olitzki, 1948). Other carbohydrates in addition to gastric mucin have a similar but lesser effect (Olitzki, 1948).

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* Senior Research Fellow, United States Public Health Service.

The results with bacteria suggested a possible usefulness of this technique with the fungi. Campbell and Saslow (1950) have reported recently that lethal infections in mice result when *Histoplasma capsulatum* suspended in gastric mucin is injected intraperitoneally. Meyer and her co-workers (1950) state that in mice intraperitoneal nodules are regularly produced by *Actinomyces bovis* suspended in gastric mucin. Without this agent, lesions are not constantly produced or are of a minor character.

METHOD

The organisms used were obtained from actively growing cultures on appropriate mediums. They were gently triturated in a sterile mortar and suspended, then centrifuged. A measured amount of the packed organisms was suspended in either 5% gastric mucin or normal saline solution to the desired volumetric concentration. Wherever possible the yeast-like phase of the organism was used because of the ease in preparing uniform suspensions. The gastric mucin solution was prepared according to the method of Miller (1935).

All the mouse studies were done in young male Swiss mice. One-half cc of the mucin suspension was injected intraperitoneally into each mouse. Saline suspensions served as controls. The chick embryo was briefly studied.

RESULTS

Mice.—Table 1 shows the number of deaths within 10 days for the various fungi studied. The concentrations used were arbitrary. Further study will be necessary to determine the minimum lethal dose for each organism.

Injection of 0.5 cc. of plain gastric mucin solution into 24 mice gave no mortality. There was no gross or micro-

scopic evidence of inflammation when the mice were examined after 10 days.

Nocardia asteroides was most extensively studied. With gastric mucin suspensions, a concentration of 1:50 by volume, or stronger regularly caused death. Saline suspensions gave no mortality even in concentrations of 1:10 by volume. Peritoneal nodules were, however, produced. A concentration of 1:200 of *N. asteroides* in saline produced no gross evidence of peritoneal involvement. A similar concentration in gastric mucin produced an extensive infection with many small nodules scattered throughout the peritoneal cavity and adhesions around the liver, stomach and spleen. In all the surviving animals there was a gradual regression in the size of the nodules, and after about 8 weeks, there was complete resolution. The organisms could be uniformly cultured from the nodules. Histologic sections showed Gram-positive filamentous rods in the center of the suppurative lesions. It was evident that nonlethal concentrations, when mixed with mucin, were greatly potentiated in the production of infection.

TABLE 1.—*Mortality in 10 days in mice following intra-peritoneal injection of suspensions of various fungi in saline and in gastric mucin.*

Organism	Concentration*	5% gastric mucin†	Saline†
N. asteroides	1:20	32/32	1/32
" "	1:40	17/18	0/18
" "	1:100	0/18	0/24
B. dermatitidis (yeast phase)	1:100	12/12	1/12
C. immitis	1:100	28/28	3/24
" "	1:300	12/12	0/12
S. schenckii (yeast phase)	1:100	3/6	0/6
" " (mycelial phase)	1:100	1/12	0/12
C. neoformans	1:100	18/18	4/18
C. albicans	1:100	11/12	0/12
" "	1:500	17/18	0/18
H. capsulatum (yeast phase)	1:100	6/6	1/6
" " (yeast phase)	1:200	2/5	0/5
" " (mycelial phase)	1:100	5/12	3/12
P. brasiliensis (mycelial phase)	1:500	0/4	0/4
H. pedrosoi	1:100	0/12	0/12
H. compactum	1:100	0/8	0/8
P. verrucosa	1:100	0/8	0/8
H. parvum	1:100	0/4	0/4
A. bovis	1:50	2/18	0/18

* Volumetric.
† Numerator equals number of deaths; denominator equals number of mice injected.

In a concentration of 1:100, the yeast phase of *Blastomyces dermatitidis* caused death within 10 days when injected with mucin. The control injections yielded only one death in 12 animals. Necropsies on the mucin injected mice showed a massive involvement of the peritoneal cavity with large nodules and plaques covering the liver and spleen. Numerous small nodules were present in the omenta and mesentery. The animals given organisms suspended in saline showed only a few scattered nodules. Microscopic sections revealed the presence of many more organisms in the animals receiving gastric mucin. The lesions are well developed in one week. This is true of practically all the fungi studied. The earliest lesions are diffuse, whitish plaques solidly encasing the major viscera. Enormous numbers of organisms are found in these plaques. Studies are under way to determine the minimum number of organisms by actual count which will produce lesions and death.

Coccidioides immitis also produced 100% mortality when a 1:100 concentration in gastric mucin was injected. Only three out of 24 control animals died with this dosage. A second series was done using a 1:300 concentration. All of the gastric mucin animals were dead in 8 days, while none of the saline controls succumbed. Autopsy findings were similar to those obtained with *B. dermatitidis*. Endosporulating organisms were found in the diseased tissues of all the mice but were far more plentiful in those receiving the gastric mucin. With mucin, the study of the parasitic phase in tissue is greatly facilitated.

Gastric mucin suspensions of the yeast phase of *Sporotrichum schenckii* in a 1:100 concentration caused death in

three out of 6 animals within 10 days. There was no mortality in the control animals. The mycelial phase of *S. schenckii* in a 1:100 concentration in mucin caused death in only one out of 12 animals. The gross appearance on necropsy was the same as with *B. dermatitidis* and *C. immitis*.

A group of mice given a 1:100 concentration of *Cryptococcus neoformans* in gastric mucin died in 6 days. The control series showed 4 deaths in 18 after 14 days. No abdominal involvement was noted with either group, but the brain tissues of the mice receiving mucin suspensions were gelatinous. The infection was far more extensive in the mice injected with mucin; its use shortens the survival time and makes death more certain.

Striking results were obtained with *Candida albicans*. Ordinarily, this organism is pathogenic only for the rabbit and the chick embryo. Of 30 mice given gastric mucin injections, 28 were dead in 48 hours. No gross pathology was noted intraperitoneally in these animals, but the organism could be readily cultured. Evidently, death occurs too rapidly for gross lesions to develop. None of the control animals had succumbed after 14 days, although they did show occasional peritoneal nodules. In gastric mucin, *C. albicans* is a highly virulent organism for the mouse. Studies with other species of *Candida* are being undertaken.

The yeast phase of *H. capsulatum* in a 1:100 concentration in gastric mucin produced a fatal infection within 10 days in all the mice. One of 6 control animals died. When a 1:200 concentration was used, two of 5 animals receiving mucin suspensions died. None of the control animals died. No nodules were found in any of the mice, but those animals injected with gastric mucin uniformly had marked hepatomegaly and splenomegaly with numerous organisms present histologically. The control mice showed little or no organ enlargement, and only a few organisms could be seen on microscopic examination. The results with *H. capsulatum* confirm the report of Campbell and Saslow (1950). The mycelial phase of *H. capsulatum* in a 1:100 concentration gave 5 deaths out of 12 mice in 10 days, when the organisms were suspended in gastric mucin. The control group had 3 deaths in 12 animals. In contradistinction to the yeast phase, however, small miliary nodules were noted throughout the peritoneal cavities of the animals receiving mucin suspensions.

A small group of mice were inoculated with a 1:500 suspension of *Paracoccidioides brasiliensis* prepared from the mycelial phase. Neither the gastric mucin nor the saline suspensions caused any mortality. Autopsies showed, however, that the gastric mucin animals had thin whitish plaques covering the liver and spleen with definite small nodules within these organs. The organisms were readily recovered in the yeast phase on culture. There was no gross or microscopic evidence of involvement of the controls.

Hormodendrum pedrosoi, *Hormodendrum compactum*, and *Phialophora verrucosa* in a 1:100 concentration gave no mortality. The gastric mucin injections produced large nodules and a thick matting on the liver and spleen. The saline injections gave only a few very small nodules. The number of organisms seen on histologic section was much greater in the mucin injected animals.

Haplosporangium parvum, a parasite of desert mice which may be related to *C. immitis* (Ashburn and Emmons, 1945), was injected in a 1:100 concentration. No mortality was observed in either group of mice, but numerous nodules were found in the mucin injected mice. Only a few were seen in the control animals.

The results with *A. bovis* have been disappointing. Three different strains have been employed. Two of these were isolated from human infections and the other from a cow. No abdominal lesions were observed even in high concentrations. There was no mortality. This is contrary to the findings of Meyer (1950). Strain differences of the organisms or the host may account for this discrepancy.

Other fungi which only occasionally cause human death, such as species of *Mucor*, *Aspergillus* and *Penicillium* will also be studied. This will be the subject of another report.

Chick embryo.—Circumscribed nodules and plaques, usually limited in number, develop on the chorio-allantoic membrane after inoculation of certain of the fungi. Included in this group are *H. capsulatum*, *B. dermatitidis*, and *S. schenckii*. The membrane infections with these organisms are not fatal.

Suspensions of the yeast phase of these three organisms were made up to a volumetric concentration of 1:200 in gastric mucin. One-tenth of one cc of each suspension was dropped onto the chorioallantoic membrane. Twenty-four embryos were used for each organism, 12 of which received the suspension in mucin. The remainder were injected with saline suspensions. In no instance was there evidence of lowering of host resistance. The embryos survived in all cases. Lesions were not more numerous with gastric mucin.

The result was not unexpected. The chick embryo has no definite means of destroying micro-organisms. Practically all the systemic fungi will reproduce a variable amount of infection of the chorio-allantoic membrane. When only a few lesions develop, this is probably not an expression of resistance, but implies that the host tissue is not a satisfactory biological substrate for the development of the fungus. Gastric mucin is presumed to act by protecting the organisms against the defensive activities of the host. When the defense reaction is minimal, as in the chick embryo, this effect is not to be anticipated.

DISCUSSION

The course of experimental infection with various fungi is markedly altered when the organisms are suspended in mucin. Death may be produced with fungi which otherwise are not lethal. Massive and rapidly developing infections can be produced by using sublethal infective doses. Animal species ordinarily resistant to infection may be rendered susceptible, viz., mouse infections with *C. albicans*.

The phenomenon of lowering of host resistance with gastric mucin lends itself to the following purposes in medical mycology:

1. Chemotherapeutic studies: the effect of a chemotherapeutic agent in preventing or delaying death can be evaluated.

2. Diagnostic tool: human biopsy tissue suspected of harboring a fungus may be ground up and suspended in 5% gastric mucin for injection into animals for diagnostic purposes. It has been possible in the present study to show that a small amount of mouse tissue infected with *B. dermatitidis*, when so prepared, yielded peritoneal lesions on reinjection into mice. No lesions resulted when the suspension of tissue was made up in saline. This technique may prove particularly valuable with human tissue in which organisms are sometimes quite scarce.

3. The mouse becomes suitable as a susceptible host for all the deep mycoses. Larger and more expensive animals such as the rabbit are not necessary.

4. The yeast phase of the dimorphic organisms such as *H. capsulatum*, *P.*

brasiliensis, etc., may be rapidly secured by inoculating mice and making cultures on blood agar at 37 C 5 to 7 days after infection. The conversion of the mycelial to the yeast phase is sometimes difficult by purely cultural methods. In such instances, animal passage is the expedient method. With gastric mucin, the time required to accomplish this is shortened.

SUMMARY

The resistance of mice was found to be markedly reduced to systemic fungus infections when the fungus is suspended in 5% gastric mucin. Fatal infections were uniformly produced with most of these organisms. With others, the extent and rapidity of infection was greatly increased.

Gastric mucin had no effect when the suspensions of fungi were placed on the chorio-allantoic membrane of the chick embryo.

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VITAMIN REQUIREMENTS OF *NEISSERIA*

JOHN L. NEMES, MICHAEL J. PELCZAR, JR., AND RAYMOND N. DOETSCH
From the Department of Bacteriology, University of Maryland, College Park, Maryland

Although numerous studies on nutritional requirements for *Neisseria gonorrhoeae* and *Neisseria meningitidis* have been made, our knowledge of the specific requirements of other members of this genus is meager. Only two reports on the requirements of "nonpathogenic" *Neisseria* have come to our attention.[1,2] The present study was undertaken to determine the vitamin requirements of a large collection of *Neisseria* species other than the pathogens.

EXPERIMENTAL

Cultures.—The cultures of *Neisseria* used in this study were isolations from human throats and from the pharyngeal region of guinea pigs. Several strains were received through the courtesy of the American Type Culture Collection, National Collection of Type Cultures, Army Medical Department Research and Graduate School, and the Department of Bacteriology of Temple University.

A total of 57 cultures, comprising 10 strains of *N. catarrhalis*, 10 strains of *N. sicca*, 8 strains of *N. perflava*, 2 strains of *N. flava*, 3 strains of *N. flavescens*, and 24 "guinea pig" strains were screened for their vitamin requirements.

TABLE 1.—*Composition of the basal medium.*

Casein hydrolyzate, acid-hydrolyzed, vitamin free	0.5 g
Glucose, CP	0.2 g
l Cysteine, hydrochloric acid	0.1 mg
Salt solution A*	2.0 ml
Salt solution B†	2.0 ml
Distilled water to make	100.0 ml

* Solution A was composed of disodium acid phosphate (anhydrous) 3.0 g, potassium dihydrogen phosphate (Sorensen's) 1.0 g, distilled water 100.0 ml.
† Solution B was composed of iron sulfate 0.05 g, manganese chloride 0.05 g, magnesium sulfate 0.50 g, sodium chloride 5.0 g, distilled water 100.0 ml.

Screening for vitamin requirements.—Vitamin requirements were determined by the conventional method of growing each culture in the basal medium (table 1) supplemented with the vitamins listed below, followed by various omissions.

The vitamin requirements were confirmed by serial subcultivation of the organisms through 5 transfers in a medium containing only the vitamin or vitamins suspected of being essential for growth of the strain under test. Growth was measured turbidimetrically with a Fisher AC electrophotometer using a 425 B filter.

Vitamins employed and their final concentrations in the basal medium included meso-inositol, 5 μg per ml; choline chloride, 2.5 μg per ml; calcium D-pantothenate, niacin, pyridoxine, riboflavin, and thiamine, each in 1.0 μg per ml; p-aminobenzoic acid, 0.5 μg per ml; pteroylglutamic acid, 0.05 μg per ml; biotin, 0.01 μg per ml.

RESULTS

The vitamin requirements of 57 cultures of *Neisseria* as determined in this investigation are presented in table 2.

Measurement of growth stimulation to graded amounts of biotin revealed maximum response with a concentration of 10^{-4} to 10^{-5} μg per ml of medium. For niacin, maximum response was given with 10^{-1} to 10^{-2} μg per ml.

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In general, growth in the various supplemented casein hydrolyzate mediums was not optimal, since more rapid and profuse growth resulted in a peptone medium, such as trypticase soy broth.

From the results presented, it appears that the "nonpathogenic" *Neisseria* are no less exacting with respect to vitamins than are the pathogens. As a matter of fact, some of the synthetic mediums described for the cultivation of meningococci[3,4] proved inadequate for the growth of many of the cultures used in this study. This finding is substantiated by a recent report[2] that *N. catarrhalis* would not grow in a chemically defined medium which was adequate for growth of the meningococcus, unless supplemented with additional amino acids. In a sense this seems paradoxical, since in general practice "enriched" mediums are recommended for the pathogenic *Neisseria*, all other species of the genus being presumably capable of growth on less complex natural mediums.

SUMMARY

Fifty-seven cultures of "nonpathogenic" *Neisseria* were screened for their vitamin requirements using a vitamin-free casein hydrolyzate basal medium. Biotin and/or niacin were required by 47 cultures; the remaining 10 were capable of growing in the absence of added vitamins, but 3 of these were stimulated by the addition of biotin. In this respect, i.e., vitamin requirements, the nonpathogenic *Neisseria* are not as simple as implied by many "textbook" descriptions.

Growth of cultures in the casein hydrolyzate medium was inferior to that supported by a natural medium such as trypticase soy broth.

TABLE 2.—*Summary of vitamin requirements of nonpathogenic* Neisseria *spp. as determined in a vitamin-free casein hydrolyzate medium.*

	Number of strains	Strain requirements					Requirements in addition to biotin and niacin
		No vitamins	Biotin	Biotin +niacin	Biotin stimulatory	Niacin	
N. catarrhalis	10	6	4		3		
N. sicca	10		8	2			
N. perflava	8		8				
N. flava	2		2				
N. flavescens	3		3				
Unclassified*	24	4	2	13		2	3†

* Strains isolated from the pharyngeal region of guinea pigs, nonfermentative, pigmented, serologically distinct from *N. flavescens*.

† Strains which grew only when thiamine, pyridoxine and Ca-D-pantothenate were added to basal medium containing biotin and niacin.

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STUDIES ON *PLASMODIUM GALLINACEUM* IN VITRO

I. A METHOD FOR MAINTENANCE OF THE ERYTHROCYTIC PARASITE IN VITRO

D. JANE TAYLOR, JOSEPH GREENBERG, EDWARD S. JOSEPHSON, AND AMAR P. RAY*

From the Microbiological Institute of the National Institutes of Health, Public Health Service, Bethesda 14, Maryland

Since the early work of Bass and Johns[1] on the cultivation of malarial parasites, there have been many improvements in mediums and techniques culminating in the successful cultivation of the erythrocytic forms of *Plasmodium lophurae* by Trager[2–4] and *Plasmodium knowlesi* and *Plasmodium vivax* by Geiman and co-workers.[5,6] The literature on in vitro cultivation of plasmodia has been reviewed by these authors. The erythrocytic forms of *Plasmodium gallinaceum* were successfully maintained by Hawking[7] by a method differing from that described in this report.

The purpose of this paper is to report a simple method for maintenance of the erythrocytic parasites of *P. gallinaceum* in vitro for periods of 24 hours. The method has been very useful in the study of the physiology of the parasites and especially, the effect upon them of chemotherapeutic agents.

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The authors gratefully acknowledge the assistance of Dr. Ralph W. McKee in procuring rocker-dilution boats and in demonstrating the techniques for the in vitro cultivation of plasmodia.

* World Health Organization Fellow. Present address: Malaria Institute of India, Delhi, India.

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EXPERIMENTAL

The medium used was heparinized or citrated blood obtained by heart puncture from New Hampshire Red chickens varying in age from 8 to 24 weeks and of both sexes. Rocker-dilution boats identical with those described by Geiman et al[5] were used as containers. To each sterile boat were added 4.4 ml of chicken blood, 0.1 ml of penicillin in 0.85% sodium chloride (5000 units of crystalline sodium penicillin G) and 0.5 ml of parasitized chick blood, diluted with 0.85% sodium chloride to contain a desired number of parasites. Donors were selected with approximately 60% of their erythrocytes parasitized. Such chicks usually have about 1.5×10^6 erythrocytes per cmm of blood. One-half ml of the blood of such chicks contains approximately 5×10^8 parasitized erythrocytes. The boats were then rocked on their long axis for various lengths of time at a rate of 17 cycles per minute inside an incubator maintained at 37 C.

Two methods of assaying survival of parasites were employed: (a) Inoculation of an aliquot of the cultures into susceptible chicks and determining the period before detection of parasitemia (prepatent period) and (b) direct examination of fixed and stained parasites. The former method has the ad-

vantage of enumerating only those parasites which are capable of reproduction in the vertebrate host, a quality which cannot be determined definitely by examining stained preparations.

P. gallinaceum exhibits relatively little variation in rate of reproduction in the chick, as determined by prepatent period and subsequent growth curves. As seen in figure 1, which summarizes the data of 39 experiments involving 269 chicks, the prepatent period decreases logarithmically with the number of parasites inoculated intravenously. Thus a 90% reduction in numbers of parasites inoculated resulted in a mean 1.3 day increase in prepatent period. The standard errors of each of the means, which reflect both the variations within groups of chicks as well as that between groups inoculated at different times, are relatively small and consistent throughout most of the range of inoculums.

Immediately after preparation of the cultures and at intervals thereafter (usually 24 and 48 hours), the cultures were tested for the numbers of viable parasites present. For this purpose one-week-old chicks were inoculated intravenously with 0.1 ml of the culture. Blood smears of the chicks were examined for parasites daily, beginning, usually, the day after inoculation and continuing until parasites were detected on two consecutive days, or, if negative, for 14 days. Smears were considered negative if no parasites were detected in 30 fields of 100 erythrocytes each.

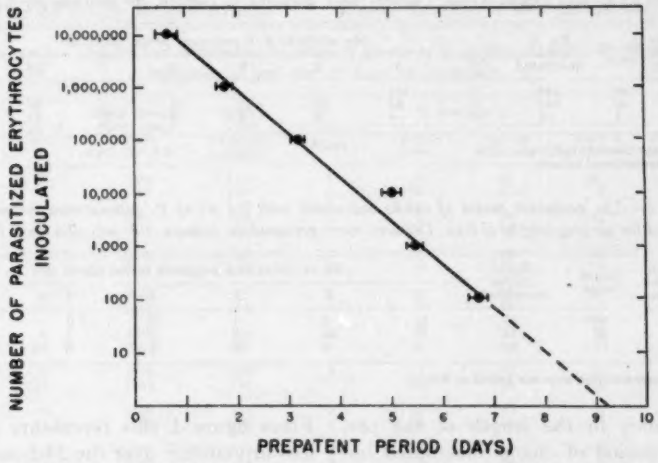

FIG. 1.—Relationship between size of inoculum and prepatent period in chicks.

Two series of experiments were performed using the above technique. In one series (20 experiments) the cultures were prepared to contain 10^5 parasitized erythrocytes per 0.1 ml and in the other (22 experiments) 10^7 parasites per 0.1 ml.

In the series with 10^6 parasitized erythrocytes per 0.1 ml, one experiment was done in quintuplicate, eight in triplicate and eleven using single cultures. A total of 40 cultures was assayed over a 24-hour period with six of them continuing for 48 hours. Fourteen of the cultures were assayed in 5 chicks each,

two in four chicks each and 24 in three chicks each. Thus, 149 chicks were inoculated immediately after preparation of the cultures (0 hour), 147 (two chicks died before detection of parasites) were inoculated after 24 hours and 18 after 48 hours.

The prepatent periods of all the chicks inoculated are shown in table 1. The of more than one day in the prepatent period of the infections in chicks inoculated with a single culture, while in 24 cultures there was no difference in the prepatent periods.

Excluding the two obvious experimental failures, there was a difference in the mean prepatent period in all the chicks inoculated at 0 and 24 hours of 0.64 day.

TABLE 1.—*The prepatent period of chicks inoculated with 0.1 ml of* P. gallinaceum *cultures incubated for varying lengths of time. Cultures were prepared to contain 10^6 parasites per 0.1 ml.*

Hours after preparation of cultures	No. of boats	No. of chicks inoculated	No. of chicks with prepatent period (days) of:							
			2	3	4	5	6	7	≧8	Mean
0	40	149	5	137	7					3.0
24	40	147	0	84	31	17	4	3	8*	3.6
48	5	18	0	0	0	8	2	3	5†	5.6

* Two experimental failures.
† One experimental failure.

TABLE 2.—*The prepatent period of chicks inoculated with 0.1 ml of* P. gallinaceum *cultures incubated for varying lengths of time. Cultures were prepared to contain 10^7 parasites for 0.1 ml.*

Hours after preparation of cultures	No. of boats	No. of chicks inoculated	No. of chicks with prepatent period (days) of:					
			1	2	3	4	5	Mean
0	30*	89	86	2	1	0	0	1.09
24	47	141	84	44	8	3	2	1.54
48	12	33	3	6	17	6	0	2.79

* Seventeen cultures were not tested at 0 hour.

consistency in the length of the prepatent period of chicks inoculated immediately after preparation of the cultures should be noted. After 24 hours of incubation the cultures produced less consistent results, there being an obvious skewness in the direction of lengthened prepatent period. In two experiments none of the chicks inoculated with parasites incubated for 24 hours developed patent infections. This accounts for the 8 chicks in the column of more than 8-day prepatent period. The inconsistency of the remainder of the data of the 24-hour cultures can be explained on the basis of loss of viability of the parasites in some of the cultures, rather than a variation in the growth rate of the parasite in the chicks of each group. For example, in only 4 cultures was there a difference

From figure 1 this represents a mean loss in viability over the 24-hour period of about 50%. However, in 16 cultures there was no difference in the mean prepatent period in chicks inoculated at 0 and 24 hours so that, presumably, within the limitations of the calculations given previously, there was no significant loss in parasite viability. In only 5 cultures was the mean prepatent period in the chicks inoculated with parasites at the end of 24 hours of incubation more than one day longer than that in chicks inoculated with those incubated for 0 hours. It is obvious from table 1 that only about 1% of the parasites present initially were infective after 48 hours.

An analysis of the data from cultures prepared to contain 10^7 parasites per 0.1 ml is shown in table 2. Again the

noticeable skewness of the 24-hour data can be explained in terms of loss of viability in some of the cultures. In this series, too, there was an over-all loss of viability of about 50%. In 16 of the cultures there was no loss of viability. This series was conducted after the previous one, and there were no frank experimental failures. Again in this series an average of about 1% of the parasites originally present in the cultures survived after 48 hours of incubation.

stages changed radically in 24 hours. Eighty-nine per cent of the mature schizonts disappeared, probably by rupturing; 92% of the rings present at the beginning of the experiment were no longer present; and about 40% of the trophozoites had disappeared. If all the mature schizonts disappeared with no reinvasion of red cells, one would expect to find 1180 parasitized erythrocytes per 10^4 erythrocytes at the end of 24 hours. Instead 1506 parasitized eryth-

TABLE 3.—*Density and distribution by stages of growth of* P. gallinaceum *in vitro at the beginning of, and after 24 hours of incubation.*

Boat	Hrs. of incubation	Mean parasite count (parasitized RBC/10^4 RBC)	Mean total parasites counted	% distribution				% loss in numbers
				Rings	Trophozoites	Young schizonts	Mature schizonts	
1	0	1680	137	1	7	45	47	
1	24	1276	120	0	7	91	2	24
2	0	2042	133	2	7	45	46	
2	24	1532	116	0	3	94	3	25
3	0	1820	123	3	11	40	46	
3	24	1244	145	0	8	91	1	32
4	0	2070	132	5	9	37	49	
4	24	1438	111	1	9	87	3	26
5	0	2020	126	5	5	55	35	
5	24	1662	113	1	3	89	7	18
6	0	1830	137	3	7	52	38	
6	24	1662	112	0	3	85	12	9
7	0	1860	134	2	15	51	32	
7	24	1600	115	0	3	91	6	14
8	0	1736	127	2	5	59	34	
8	24	1636	114	0	3	94	3	6
Mean	0	1845		3	8	48	41	
	24	1506		<1	5	90	5	19

In 8 cultures prepared to contain 10^7 parasitized erythrocytes per 0.1 ml, 5 thin smears were made immediately after preparation of the culture and 5 additional smears were made after 24 hours of incubation. After being fixed and stained with Giemsa, each slide was examined for parasite density (parasitized erythrocytes per 10^4 erythrocytes) and the numbers of each of 4 stages of parasite development (table 3).

In 24 hours there was a mean decrease in parasite density of about 19%. However, it is apparent that the parasite population was not static, since the relative proportions of parasites at various

rocytes per 10^4 erythrocytes were found, indicating that one-half of the mature schizonts lost were replaced by parasites in other stages. The replacement occurred almost exclusively in the stage of the young schizonts, of which there were considerably more after 24 hours than one would expect if all other stages were preferentially killed. It is possible that some of the parasites classified as young schizonts were old schizonts which had lost their chromatin bodies. Alternately, it is possible that some of the merozoites produced by mature schizonts invaded erythrocytes and developed to the stage of young schizonts.

The block in parasite development would then be at the stage where division of the chromatin body occurs.

By comparing these data with those obtained from inoculating the cultures into chicks, it is estimated that about 22% of the parasites seen in the slide preparations were not able to reproduce normally in the chick. On the other hand, the parasite population after 24 hours of incubation was younger than the initial population. This could result in a delay in the prepatent period of the inoculated chicks which would be wrongly interpreted as a loss in viability.

The rocking motion of the boats and the use of whole blood appear to be critical in this method. In stationary cultures parasites failed to survive more than a few hours, contrary to the findings of Hawking.[7] When whole blood was replaced by serum or plasma, parasites seldom survived 24 hours. However, washed chick erythrocytes or chick erythrocyte extracts prepared according to the method of Whitman[8] were found to be almost complete substitutes for whole blood, which agrees with the findings of Hawking.[7] The addition of glucose, p-aminobenzoic acid, ascorbic acid and methionine to the medium failed to increase viability of the parasites. Subcultures made at 24 hours did not increase survival.

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SUMMARY

A simple method for the maintenance of erythrocytic forms of *Plasmodium gallinaceum* in vitro in a whole blood medium is described. A quantitative assay of the viability of the parasites by inoculation into young chicks is presented and compared with parasite densities as obtained from stained film preparations.

After 24 hours of incubation there was an apparent mean reduction of 50% in numbers of viable parasites. However, in one-third of the cultures there was no detectable loss of viability. By the direct examination of stained film preparations the mean reduction in parasite densities after 24 hours was 19%. After 48 hours, there was a mean reduction of 99% in numbers of viable parasites.

Observations of stained film preparations indicate that the parasites failed to segment. Attempts to improve the basal medium are described.

STUDIES ON *PLASMODIUM GALLINACEUM* IN VITRO

II. THE EFFECTS OF SOME 8-AMINOQUINOLINES AGAINST THE ERYTHROCYTIC PARASITES

JOSEPH GREENBERG, D. JANE TAYLOR, AND EDWARD S. JOSEPHSON

From the Microbiological Institute of the National Institutes of Health, Public Health Service, Bethesda 14, Maryland

Certain 6-methoxy members of the 8-aminoquinolines (pamaquine, pentaquine, and isopentaquine) have been reported to be relatively inactive in vitro against *Plasmodium cynomolgi*[1] and *Plasmodium falciparum*[2] at concentrations far above those found in the plasma of adequately treated patients infected with *Plasmodium vivax*.[3–6] During our study of the in vitro effects of 8-aminoquinolines against the erythrocytic forms of *Plasmodium gallinaceum*, several 6-hydroxy-8-aminoquinolines synthesized by Drake et al[7,8] were found to be more active than the corresponding 6-methoxy derivatives. This observation renewed our interest in the 6-hydroxy-8-aminoquinolines even though they demonstrated in vivo little or no activity against tissue forms of *P. vivax*[4,9] or *P. gallinaceum*.[10]

This report deals with a comparison of the in vitro activity of some 6-hydroxy- and 6-methoxy-8-aminoquinolines and related compounds against erythrocytic forms of *P. gallinaceum*. This project was undertaken with the close cooperation of Mr. Nathan L. Drake and Miss Yolanda T. Pratt of the University of Maryland, who synthesized many of the compounds tested.

METHODS

The erythrocytic forms of *P. gallinaceum* were maintained in vitro by a method described in a previous paper.[11] For appraisal of drug activity against this parasite rocker-dilution boats were prepared as follows: To each boat were introduced 3.9 ml of heparinized chick blood; 0.1 ml of penicillin in 0.85% sodium chloride (5,000 units of crystalline sodium penicillin G); 0.5 ml of parasitized blood diluted with 0.85% sodium chloride, so that each 0.5 ml contained 5×10^8 parasitized erythrocytes; and 0.5 ml of test drug solution. The test compounds in 0.1 *N* hydrochloric acid were adjusted to pH 6.0 to 8.0 with 10 *N* sodium hydroxide immediately prior to their addition to the boats.

Each compound was first tested at a concentration of 5 mg/liter of final medium. If found active at this concentration, it was tested in twofold dilutions until a concentration was reached which produced no significant activity on the parasites. Each concentration of test drug was tested in two boats. Serving as controls for each series were two boats in which 0.5 ml of 0.1 *N* hydrochloric acid,

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adjusted to pH 6 to 8 with 10 *N* sodium hydroxide, replaced the 0.5 ml of test drug solutions. All boats were rocked at 37 to 38 C for 24 hours at 15 to 17 cycles per minute.

The numbers of viable parasites in each control boat were estimated immediately after preparation of the cultures and again at the end of 24 hours by inoculating aliquots of the cultures into susceptible hosts and determining the prepatent period.[11] In boats containing test drugs, the numbers of viable parasites were estimated only at the end of each run.

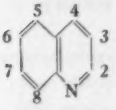

TABLE 1.—*The effect of various substituents on the 6-position of certain 8-alkylamino-alkylamino quinolines on their in vivo and in vitro antimalarial activity and toxicity in the chick.*

Compound	Substituent on position		Minimum effective concentration in vitro μg/L	Minimum effective dose in vivo mg/g b.i.d.	Maximum tolerated dose (chicks) mg/g b.i.d.	Therapeutic index
	6	8				
SN 13,457	H	$NH(CH_2)_3N(C_2H_5)_2$	>5,000	.0035	.01	3
SN 971 (pamaquine)	OCH_3	$NHCH(CH_3)(CH_2)_3N(C_2H_5)_2$	>5,000	.0006	.01	17
SN 13,276 (pentaquine)	OCH_3	$NH(CH_2)_4NHCH(CH_3)_2$	>5,000	.0001	.005	50
SN 14,993	$O(CH_2)_2OH$	$NH(CH_2)_4NHCH(CH_3)_2$	>5,000	.004	.005	1.1
SN 13,125	OH	$NH(CH_2)_3N(C_2H_5)_2$	300	.002	.010	5
DR 15,324	OH	$NH(CH_2)_4NHCH(CH_3)_2$	625	.0005	.032	64
DR 14,382	OH	$NH(CH_2)_3N(C_2H_5)_2$	300	.001	.175	175
DR 15,433	$OOCCH_3$	$NH(CH_2)_3N(C_2H_5)_2$	600	.001	.0125	12.5

The minimum effective concentration of a test drug is defined as that amount which will prevent 90% of the parasites, initially present in each boat, from reproducing when subinoculated into week-old chicks. This corresponds to a 1.3 day increase in the mean prepatent period of the infection in chicks inoculated with parasites exposed to drug over that of those inoculated with control cultures.[11]

For most of the compounds tested, the minimum effective dose in vivo against trophozoites of *P. gallinaceum* in the chick was determined by the method of Coatney and Sebrell.[12] The minimum effective dosages of the compounds for the chick were determined by Dr. N. B. Eddy by a method previously described.[12]

RESULTS

8-aminoquinolines with methoxy, oxyethanol or hydrogen in the 6-position of the nucleus were inactive in vitro at concentrations of 5000 μg per liter of culture medium (table 1). In contrast, the 6-hydroxy-8-aminoquinolines were active at 300 to 600 μg per liter. Esterification of the 6-hydroxy group did not reduce the in vitro activity (compare DR 15,382 with DR 15,433). The 6-methoxy-8-aminoquinolines were as active and in some cases more active in vivo than the 6-hydroxy-8-aminoquinolines. In vitro activity, therefore, does not correlate with in vivo activity. Furthermore, even among the 6-hydroxy compounds there was no direct correlation between in vitro and in vivo activity, although this may be the result of limitations in the antimalarial tests.

The effect of a second nuclear hydroxy substituent was studied next. As shown in table 2, the 5,6-dihydroxy-8-(isopropylaminoamylamino) quinoline (DR 15,873) was four times as active in vitro as its 6-hydroxy analogue (DR 15,324). On the other hand, 2,6-dihydroxy-8-(diethylaminopropylamino)quinoline (SN 14,184) was inactive at 5000 μg per liter in vitro and had no antimalarial activity in vivo. In this group of compounds there appears to be some correlation between in vitro and in vivo activity, since DR 15,873, which was more active in vitro than DR 15,324, was also more active in vivo.

12. Coatney, G. R. and Sebrell, W. H., in F. Y. Wiselogle 1946, A Survey of Antimalarial Drugs 1941–1945. Published by J. W. Edwards. Ann Arbor, Mich. 1: 457–462.

TABLE 2.—*The effect of a second hydroxyl group upon the in vitro and in vivo activities of 6-hydroxy-8-alkylaminoalkylamino-quinolines.*

Compound number	Substituent on position				Minimum effective concentration in vitro μg/L	Minimum effective dose in vivo mg/g b.i.d.	Maximum tolerated dose for chicks mg/g b.i.d.
	2	5	6	8			
DR 15,324	H	H	OH	$NH(CH_2)_3NHCH(CH_3)_2$	625	0.0005	0.02
DR 15,873	H	OH	OH	$NH(CH_2)_3NHCH(CH_3)_2$	150	0.00025	>0.005
SN 14,184	OH	H	OH	$NH(CH_2)_3N(C_2H_5)_2$	>5,000	>0.3	0.3+

Since hydroxyl groups in the 5 and 6 positions of the quinoline nucleus were consistently associated with direct plasmodicidal activity, the in vitro activity of a 5-hydroxy-6-methoxy-8-aminoquinoline was investigated (table 3). This compound (DR 15,895), an analogue of pentaquine, was found to be active in the same range of concentrations as the 6-hydroxy-8-aminoquinolines (table 1). In contrast the 5-chloro and 5-methoxy analogues of pentaquine were inactive at 5000 μg per liter of culture medium.

TABLE 3.—*The effect of other nuclear substituents upon in vitro activity of 6-methoxy-8-alkylaminoalkylamino-quinolines.*

Compound number	Substituent on position			Minimum effective concentration in vitro μg/L
	5	6	8	
SN 13,276	H	OCH_3	$NH(CH_2)_4NHCH(CH_3)_2$	>5,000
DR 15,884	Cl	OCH_3	$NH(CH_2)_4NHCH(CH_3)_2$	>5,000
DR 15,332	OCH_3	OCH_3	$NH(CH_2)_4NHCH(CH_3)_2$	>5,000
DR 15,895	OH	OCH_3	$NH(CH_2)_4NHCH(CH_3)_2$	600

Finally, the in vitro activity of 6-hydroxy-quinolines with degraded 8 position side chains was studied (table 4). These tests showed that when the terminal alkylamino group of this side chain was absent, activity was markedly reduced. When the side chain consisted solely of a primary amine, the compound was inactive within the limits of this test. A compound with only the terminal alkyl group absent was not available for testing.

DISCUSSION

It has been shown in this report that the 6-methoxy-8-aminoquinolines, pamaquine and pentaquine, which are very active against *P. gallinaceum* in the chick, are relatively inactive against this parasite in vitro. This observation parallels those of other investigators who found these compounds relatively inactive in vitro against other species of plasmodia.[1,2] It is likely, therefore, that the antiplasmodial activity of these compounds is associated with metabolic intermediates. In contrast, corresponding 6-hydroxy-8-aminoquinolines have been shown to be highly active in vitro, as well as in vivo, against *P. gallinaceum*. Furthermore, it was found that 5,6-dihydroxy-8-(isopropylaminoamylamino)quinoline (DR 15,873) was four times as active in vitro against this parasite as 6-hydroxy-8-(isopropylaminoamylamino)quinoline(DR15,324).

TABLE 4.—*The effect of degradation of the 8-position side chain of 6-hydroxy-8-aminoquinolines on in vitro antimalarial activity.*

Compound number	8-position substituent	Minimum effective concentration in vitro μg/L
DR 13,125	$NH(CH_2)_4NHCH(CH_3)_2$	300
DR 15,324	$NH(CH_2)_3N(C_2H_5)_2$	625
DR 15,883	NHC_4H_9	2,500
DR 15,881	NH_2	>5,000

However, not all quinolinols have direct antimalarial activity, since 2,6-dihydroxy-8-(diethylaminopropylamino)quinoline (SN 14,184) was inactive both in vitro and in vivo. Furthermore, degradation of the 8-position side chain of 6-hydroxy-8-aminoquinolines to an alkylamino group was accompanied by reduction in in vitro activity, and degradation of this side chain to the primary amine rendered the compound inactive within the limits of the test. Therefore, it appears that the activity of hydroxyquinolines in vitro against *P. gallinaceum* is a function of the number and position on the quinoline nucleus of the hydroxy groups as well as the nature of the 8-position side chain.

The question arises as to what relationship, if any, exists between the hydroxy and methoxyquinolines. Schönhöffer[13] has postulated that antimalarial activity can be expected only of those 8-aminoquinolines which can undergo biological oxidation to quinonimines and quinones. Blanchard[14] has also suggested that the 8-aminoquinolines, which have antimalarial properties, are first oxidized by the host to the 5 or 7 hydroxy derivatives and then to quinonimines. These hypotheses receive some support from our data where it is shown that the 5-hydroxy-6-methoxy-8-(isopropylaminoamylamino) quinoline was at least ten times as active in vitro against *P. gallinaceum* as 6-methoxy-8-(isopropylaminoamylamino)quinoline. In addition, the oxidation of 6-hydroxy-8-(isopropylaminoamylamino) quinoline to the 5,6-dihydroxy compound enhanced its direct antimalarial activity. However, it should be pointed out that, according to Drs. Pratt and Drake, who synthesized these 5-hydroxy quinolines, these compounds are extremely unstable in solution. It is, therefore, a moot question as to whether it is the 5-hydroxy-quinolines or their degradation products which are responsible for the in vitro antimalarial activity. It should also be pointed out that so far as we know no one has isolated any 5-hydroxy-quinolines from animals treated with 6-methoxy or 6-hydroxy-quinolines.

Although the possibility exists that the 6-methoxy-8-amino-quinolines are transformed in vivo to 6-hydroxy-8-aminoquinolines, there are other facts which suggest that this does not occur. For example, while certain 6-methoxy-8-aminoquinolines can eradicate sporozoite-induced *P. vivax* infections in man, presumably by killing the fixed tissue forms of the parasite, the corresponding 6-hydroxy-8-aminoquinolines cannot.[4] In addition, it has been found in this laboratory that certain 6-methoxy-8-aminoquinolines are active against late tissue forms of *P. gallinaceum*,[15] while the corresponding 6-hydroxy-8-aminoquinolines are not (unpublished data). Since Tonkin[16] has found that the 6-methoxy-8-aminoquinoline, pamaquine, was inactive against tissue forms of this parasite in vitro, it can be assumed that the activity of 6-methoxy-8-aminoquinolines in vivo is attributable to their biological degradation products. It seems obvious that the active degradation product of pamaquine cannot be a 6-hydroxy-8-aminoquinoline or any degradation product of this group of compounds, otherwise these also would be active against the fixed tissue parasites. It must be concluded, therefore,

13. Schönhöffer, F. 1942, Ztschr. f. physiol. Chem. **274**: 1–8.
14. Blanchard, K. C., in F. Y. Wiselogle 1946, A Survey of Antimalarial Drugs 1941–1945. Published by J. W. Edwards, Ann Arbor, Mich. **1**: pp. 129–138.
15. Greenberg, J., Trembley, H. L. and Coatney, G. R. 1950, Am. J. Hyg. **51**: 194–199.
16. Tonkin, I. M. 1946, Brit. J. Pharm. & Chemotherap. **1**: 163–173.

that the mode of action of the 6-hydroxy-8-aminoquinolines against plasmodia is to some extent different from that of the 6-methoxy-8-aminoquinolines or their degradation products. It would seem that the 6-hydroxy-8-aminoquinolines, which are relatively new and untested compounds, would be worthy of trial in infections other than malaria, especially in view of the fact that they are considerably less toxic than their 6-methoxy analogues.

SUMMARY

A series of 8-aminoquinolines was examined for activity in vitro and in vivo against the erythrocytic parasites of *Plasmodium gallinaceum*. The 6-hydroxy members tested were at least 2 to 33-fold more active in vitro than their 6-methoxy counterparts. In vivo the 6-hydroxy-8-aminoquinolines were less toxic to the chick and were, in general, somewhat less active as plasmodicidal agents than the 6-methoxy-8-aminoquinolines. Introduction of a hydroxyl group in position 5 enhanced the antiplasmodial activity of the 6-methoxy- and 6-hydroxy-8-aminoquinolines in vitro and of the 6-hydroxy-8-aminoquinolines in vivo. The effects of other substituents on the quinoline nucleus and modifications in the 8-amino side chains are discussed.

ON THE TOXIC AND ALLERGIC MANIFESTATIONS CAUSED BY THE TISSUES AND FLUIDS OF ASCARIS

III. HYPERSENSITIVITY THROUGH INFECTION IN THE GUINEA PIG

J. F. A. SPRENT

From the Department of Parasitology, Ontario Research Foundation, Toronto 5, Canada

In a previous paper (Sprent, 1950), it was shown that guinea pigs infected with ascaris larvae are rendered hypersensitive to certain ascaris extracts, as shown by the occurrence of anaphylactic shock when these extracts are injected intravenously. A preliminary attempt was made to separate the substance responsible for the reaction by treatment of the original whole worm extract with various protein precipitants. The present paper records some further investigations along these lines, special attention being paid to the anaphylactic activity of adult and larval tissues and metabolic products.

METHODS

Ascaris eggs were removed from the uterus of adult pig ascaris obtained from the slaughter house, they were cultured in moist charcoal for 40 days and, when required, the eggs were washed off the charcoal in tap water. A dilution count was made of the approximate number of eggs per unit volume and the volume adjusted so that one-half cc contained approximately 5000 eggs. Care was taken to give fairly uniform doses to each animal by shaking the suspension of eggs constantly while quickly withdrawing one-half cc into a pipette fitted with a rubber bulb and marked so that one-half cc could be withdrawn at once. Before the fluid was withdrawn from the suspension, a rubber stopper with a hole bored in it was inserted between the incisor teeth of the guinea pig and the fluid was then immediately introduced through the cork. Establishment of infection was confirmed by the occurrence of dyspnoea on the 8th day of infection. Guinea pigs infected with 600 larvae of *Trichinella spiralis* were kindly supplied by Dr. E. Kuitunen-Ekbaum, of the University of Toronto. The various whole worm extracts were prepared in a similar way to that already described (Sprent, 1949), except that the fresh material was homogenized and centrifuged before lyophilization and only the supernatant fluid was used. When required for injection the material necessary for one day was weighed, dissolved in sterile saline, adjusted to pH 7.0, homogenized and centrifuged to ensure the absence of any particulate matter. In all cases the saphenous vein was used for intravenous injection. The metabolic products of adult ascaris were obtained by selecting small active worms in the slaughter house and placing them immediately in a thermos flask of physiological saline at 37 C. In the laboratory they were placed in dialyzing tubing filled with saline, and the tubing was placed in a liter flask through which saline at 37 C was continually perfused. In this way, any dialyzable excretory products were removed from the worms, while non-dialyzable excretory products were retained. After 4 days the material within the tubing was centrifuged, dialyzed against distilled water and then lyophilized. Larval metabolic products were obtained by mincing and centrifuging the lungs of heavily infected guinea pigs on the 8th day after infection; the supernatant fluid was lyophilized. The various chemical extracts used in these experiments were obtained as previously described (Sprent, 1950), any modifications being described under experiment 3. Table 1 shows the abbreviations used for various extracts.

The nitrogen content of extracts A_s3 and A_s3p were determined by the micro-Kjeldahl method through the kind co-operation of Mr. L. A. Campbell, of the Biochemistry Department of the Ontario Research Foundation. The extracts (A_s3 and A_s3p) were found to contain 1.44 g and 0.47 g per 100 g respectively. The nitrogen content of other extracts prepared in a similar way to those used in the present experiments were shown in a previous publication (Sprent, 1950).

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EXPERIMENTAL

Experiment 1.—Guinea pigs were infected with 5000 ascaris eggs and 14 days later, were injected intravenously with 0.01 g of various isolated tissues of the adult worm suspended in 1 cc of physiological saline. The results are shown in table 2. It is evident that all the isolated tissues will produce anaphylaxis in infected animals.

Experiment 2.—This experiment was similar to experiment 1 except that the isolated tissues had been previously treated with 1 volume of 10% trichloracetic acid, dialyzed and lyophilized. A certain amount (0.01 g) of the resulting extract was dissolved in saline and intravenously injected into infected guinea pigs. The results are shown in table 2. It appears that the various ascaris tissues differ in their anaphylactic activity after protein has been removed by trichloracetic acid. Deproteinized ovary, body fluid and intestine evidently contain anaphylaxis-producing substances, while cuticle and muscle do not. The failure of muscle tissue to produce anaphylaxis is of importance, because this tissue contains considerable amounts of polysaccharide in the form of glycogen. It appears from later experiments that the polysaccharide fraction of ascaris may be involved in the production of anaphylactic shock, and this result indicates that glycogen itself is not active. Tissue extracts after removal of proteins varied in their clarity. Muscle was deeply opalescent, ovary slightly opalescent, body fluid, intestine and cuticle were clear. The opalescence was probably due to the presence of glycogen.

Experiment 3.—Guinea pigs infected 14 days previously with 2500 ascaris eggs were injected intravenously with the ascaris extracts shown in table 3. Whole aqueous ascaris extracts (A_1) produced anaphylactic shock in infected guinea pigs. The various other extracts shown in table 3 were prepared with a

TABLE 1.—*Guide to abbreviations used for various ascaris extracts.*

A_1	Supernatant fluid from crushed whole worms
A_2	Filtrate after precipitation of A_1 with 10% trichloracetic acid
A_p	Filtrate after precipitation of A_1 with 10% phosphotungstic acid
A_s	Filtrate after saturation of A_1 with ammonium sulphate
A_{1s}	Filtrate after saturation of A_2 with ammonium sulphate
A_{1w}	Precipitate after saturation of A_2 with ammonium sulphate
$A_{1w}p$	Filtrate after precipitation of A_{1w} with 10% phosphotungstic acid
A_{1p}	Filtrate after precipitation of A_2 with 10% phosphotungstic acid
A_{1d}	Filtrate after digestion of A_1 with 1% pepsin for 36 hours
A_3	Filtrate after precipitation of A_2 with 95% alcohol
A_3p	Filtrate after precipitation of A_3 with 10% phosphotungstic acid
A_s3	Precipitate after precipitation of A_3 with 95% alcohol
A_s3p	Filtrate after precipitation of A_s3 with 10% phosphotungstic acid

TABLE 2.—*Effect of different ascaris tissues on ascaris-infected guinea pigs.*

Tissue	No. of animals	Effect
(Whole tissue)		
Ovary	3	+++
Muscle	3	++
Intestine	3	(1) +++ (2) ++
Body fluid	3	+++
Cuticle	3	++
(After trichloracetic acid precipitation)		
Ovary	2	+++
Muscle*	2	−
Intestine	2	+++
Body fluid	2	++
Cuticle	2	(1) + (1) −

+++ denotes fatal anaphylactic shock, ++ severe dyspnoea and coughing, + slight dyspnoea and − no symptoms. When all animals in a group did not react similarly, the number of animals giving each degree of shock is indicated in parenthesis.

* This material was obtained by precipitation in 2 volumes of alcohol after trichloracetic acid precipitation.

TABLE 3.—*Effect of various ascaris extracts on ascaris-infected guinea pigs.* Dose: 0.01 g in physiological saline.

Extract	No. of animals	Effect
A_1	6	+++
A_2	6	+++
A_s	5	+++
A_p	9	(3) +++ (3) ++ (3) +
A_{1d}	4	(3) +++ (1) ++
A_{1p}	6	+
A_s1	6	+++
A_s2	2	+++
A_s3	6	+++
A_s3p	8	(4) +++ (4) ++
A_3	6	+++
A_3p	1	−
A_{1s}	6	+++
A_{1w}	2	+++
$A_{1w}p$	4	(2) +++ (2) ++

view to discovering, in a broad sense, what chemical substances are involved in this reaction. Three different protein precipitants were used. Trichloracetic acid failed to remove the activity of the parent extract A_1 (see extract A_2), and saturation with ammonium sulphate also failed (A_s). When these two precipitants were both used on the same sample, there was also no removal of activity (A_2s). Phosphotungstic acid used alone caused some decrease of activity (A_p); and when it was used following treatment with trichloracetic acid, there was definite decrease, but not complete removal, of activity (A_2p).

Digestion of extract A_2 with 1% pepsin-hydrochloric acid mixture for 36 hours failed to alter its activity (A_2D). It appeared that none of the processes intended to remove proteins from the parent extract altered the anaphylactic effect, with the exception of successive precipitation with trichloracetic and phosphotungstic acids. Following saturation of extract A_2 with ammonium sulphate the opalescence of the solution was removed, and a precipitate (A_2W) was obtained and purified by successive reprecipitations in alcohol from aqueous solution. This material was found to be active in causing anaphylactic shock, and this effect was not removed by precipitation with one volume of 10% phosphotungstic acid. The opalescence of extract A_2 was also completely removed by pouring it into 2 volumes of 95% ethyl alcohol to which a small amount of sodium chloride had been added. The precipitate, which will be referred to as a polysaccharide fraction, was purified by one, two or three successive reprecipitations in alcohol from aqueous solution (A_s1, A_s2, A_s3), and found to be active in causing anaphylaxis. This effect was not removed by phosphotungstic acid (A_s3p). After treatment with phosphotungstic acid, extracts A_2W and A_s3 were dialyzed and lyophilized before injection. The filtrate after precipitation of extract A_2 in alcohol (extract A_3) was subjected to vacuum distillation at 45 C to remove alcohol and lyophilized. It was found to be active in causing anaphylaxis, but its effect was entirely removed by treatment with phosphotungstic acid (A_3p). These results appeared to indicate that there were two fractions, probably non-protein, concerned in the production of anaphylactic shock. One was insoluble in alcohol and not affected by phospho-

TABLE 4.—*Effect of decreasing doses of certain ascaris extracts on ascaris-infected guinea pigs.*

Extract	Amount injected (g)	No. of animals used	Effect
A_1 (whole adult)	0.005	(2)	+++
A_1	0.001	(2)	+++
A_1	0.0005	(1)	++
whole larvae	0.005	(1)	+++
	0.001	(1)	+++
	0.0005	(1)	++
A_1s	0.005	(2)	+++
A_1s	0.002	(2)	+++
A_1s	0.001	(6)	+++
A_1s	0.0005	(3)	+++
A_1s	0.0002	(1)	+++
A_s3	0.01	(2)	+++
A_s3	0.005	(2)	(1) +++ (1) ++
A_s3	0.002	(1)	+
A_s3	0.001	(4)	(1) ++ (2) + (1) −
A_s3	0.0005	(1)	+
A_s3p	0.01	(2)	+++
A_s3p	0.005	(2)	(1) +++ (1) ++
A_s3p	0.001	(2)	(1) ++ (1) +

tungstic acid. Such a material was yielded by extracts A_s3 and A_2W and it seems likely that the material is either a polysaccharide or closely associated with polysaccharide. The other active substance was soluble in alcohol, and its effect was removed by phosphotungstic acid. Such a substance was yielded by extract A_3. Probably extract A_2s contained a similar substance.

Experiment 4.—In order to investigate the relative potency of adult (A_1) and larval extracts, together with those believed to contain polysaccharide (A_s3 and A_s3p) and those believed to be free of polysaccharide (A_2s), infected guinea pigs were injected intravenously with decreasing amounts of these extracts as shown in table 4. From this table it was apparent that the "polysaccharide" fraction (A_s3) was evidently less potent than the fraction present in extract A_2s, but that its potency was not appreciably diminished by precipitation with phosphotungstic acid (A_s3p). Weight for weight, the extract A_2s was the most potent and actually more potent than the original extract A_1.

Experiment 5.—Four series of 3 guinea pigs were infected respectively with 1200, 600, 300 and 150 ascaris eggs. Fourteen days later, they were injected intravenously with the extracts shown in table 5. The whole extract (A_1), the polysaccharide fraction (A_s3) and the fraction soluble in saturated ammonium sulphate (A_2s) caused anaphylactic shock in all groups. This indicates that sensitivity through infection may arise through a very light infection, and that the effect produced by the various extracts is not altered in intensity by the degree of infestation.

Experiment 6.—Having shown that possibly two nonprotein fractions are active in producing anaphylaxis in infected guinea pigs, it was decided to investigate whether immunologically similar substances were present in metabolic products obtained from adult and larval worms (see METHODS). Guinea pigs infected in the usual way were injected intravenously as shown in table 6. Metabolic products obtained from adult worms caused anaphylaxis, and their effect was not altered by precipitation with trichloracetic acid. There was some indication that the preparation containing larval metabolic products was also active, but removal of protein with trichloracetic acid or by peptic digestion appeared to remove from it almost all anaphylactic effect. The anaphylactic activity of deproteinized metabolic products from adult worms indicates that substances similar in their effect to those occurring in the tissues of the worms are actually excreted by the worms. If this is true of larvae as well as adult worms, then the sensitization of infected animals could perhaps be explained as resulting from an immunological response to the liberation of substances in the host tissues from the migrating larvae. Whether these substances could act as antigens, however, remained to be investigated (see experiments 7 and 8).

Experiment 7.—This experiment was an extension of experiment 6 in that it was intended to show whether any im-

TABLE 5.—*Sensitivity of guinea pigs infected with different doses of eggs.*

Extract (0.01 g)	1200 eggs	600 eggs	300 eggs	150 eggs
A_1	+++	+++	+++	+++
A_2s	+++	+++	+++	+++
A_s3	+++	+++	++	+++

TABLE 6.—*Effect of "metabolic products" of ascaris larvae and adults on ascaris-infected guinea pigs.*

Material injected	No. of animals	Effect			
Metabolic products (adult)	6	(4)	+++	(2)	++
after trichloracetic acid pptn.	4		+++		
Metabolic products (larvae)	6	(3)	++	(3)	+
after peptic digestion	5				+
after trichloracetic acid pptn.	5	(1)	+	(4)	−

munological relationship exists between substances present in the worm tissues and substances excreted by the worms. Six guinea pigs were sensitized by two intraperitoneal injections separated by one week, each injection consisting of 0.01 g of adult metabolic products in physiological saline. Fourteen days after the second injection, the animals were injected intravenously with 0.01 g of the materials shown in table 7. These results appeared to give no indication of immunological relationship between excreted metabolic products and the components of adult worms. It is possible, however, that "metabolic products" prepared in this way were not antigenic. The positive effect of "metabolic products" on these animals may have been due to sensitization to a bacterial protein.

TABLE 7.—*Effect of certain ascaris extracts on guinea pigs sensitized with adult ascaris metabolic products*

Shocking extract	Effect
A_1	(2) –
A_s3	(2) –
A_2s	(2) –
Metabolic products (adult)	(1) ++ (1) +

Experiment 8.—This experiment was designed to reveal any immunological relationships between substances present in adult tissues and substances present in larval tissues, larval metabolic products and adult metabolic products. Three series of guinea pigs were sensitized to extracts A_1, A_s3 and A_2s respectively, by two intraperitoneal injections, separated by one week, of 0.01 g of extract in 1 cc of physiological saline. Fourteen days after the second sensitizing injection, the animals were injected intravenously with 0.01 g of the materials shown in table 8. The results in table 8 showed that animals sensitized against whole adult tissues (A_1) were shocked by adult tissue extracts, adult metabolic products and ground larval tissues but not by larval metabolic products. Those sensitized against ascaris polysaccharide (A_s3) were shocked by the same materials, except that the fraction A_2s was without any effect, thus indicating that fraction A_2s contained no polysaccharide. Guinea pigs sensitized with fraction A_2s showed no effect from any of the injected tissues, indicating that though this fraction is highly potent in causing anaphylaxis in infected animals, it is not a complete antigen. The positive effect of whole larvae indicates that the body of the larvae and adult worms contain immunologically related substances, and the positive effect of whole and deproteinized adult metabolic products indicates that immunologically active substances are released by the adult worms and, furthermore, that at least some of these substances are not proteins.

Experiment 9.—In the following experiment ascaris infected guinea pigs were injected intravenously with extracts prepared from various helminths (see METHODS). A dose of 0.01 g was dissolved in 1 cc of physiological saline, homogenized and centrifuged. The in-

TABLE 8.—*Cross sensitization with three ascaris extracts.*

Sensitizing extracts	Adults			Larvae	Metabolic products		
				whole larvae	*adult*	*adult* (deproteinized)*	*larval*
	A_1	A_s3	A_2s				
A_1	+++	++	+++	+++	+++	+++	–
A_s3	+++	++	–	+++	+++	+++	–
A_2s	–	–	–	–	–	–	–

* Protein was removed by trichloracetic acid.

TABLE 9.—*Effect of various helminth tissues on ascaris-infected guinea pigs.*

Helminth	Host	No. of animals	Effect
P. maxillaris	Skunk	4	(2) +++ (2) ++
T. spiralis (infected diaphragm)	Guinea pig	4	–
T. spiralis (larvae)	Guinea pig	4	(2) + (2) –
A. columnaris	Bear	4	(1) +++ (3) ++
T. leonina	Dog	4	(3) +++ (1) ++
F. magna	Moose	2	–
E. granulosus (larval)	Moose	2	–
M. expansa	Sheep	3	–
Schizotaenia sp.	Porcupine	3	–

jections were performed 2 to 3 weeks after infection. The results are shown in table 9. It will be observed from this table that anaphylaxis followed the injection of extracts made from helminths within the same family as *Ascaris lumbricoides*, namely, *Ascaris columnaris* and *Toxascaris leonina* and also from a more distantly related nematode, *Physaloptera maxillaris*. Another nematode, *Trichinella spiralis*, which is probably even more distantly related, a trematode (*Fascioloides magna*) and three cestodes (*Moniezia expansa*, *Schiszotaenia sp* and *Echinococcus granulosus*) all failed to produce anaphylactic shock.

Experiment 10.—Guinea pigs were sensitized by two intraperitoneal injections of ascaris extract A_1 and A_s3, respectively, each injection consisting of 0.01 g in 1 cc of physiological saline. About two weeks after the second injection, the animals were injected intravenously with the materials shown in table 10. It will be observed from this table, that guinea pigs sensitized to whole ascaris extract A_1 showed anaphylactic shock following injection with whole worm extracts of the nearly related nematodes *T. leonina* and *A. columnaris*. Guinea pigs sensitized with ascaris polysaccharide (A_s3) showed evidence of similar sensitivity when injected with *T. leonina* and *A. columnaris*, suggesting that the polysaccharide fraction of ascaris is common to nearly related worms, but bears no immunological affinities with the components of more distantly related worms or to such substances as the Forssman polysaccharide in guinea pig kidney and to the human isoagglutinogens.

Experiment 11.—In this experiment guinea pigs infected with the nematode *T. spiralis* were used. Four weeks after infection with 600 larvae, these animals were intravenously injected with various extracts as shown in table 11. In each case 0.01 g in 1 cc physiological saline was given. The results confirm previous findings in that they indicate the sensitivity of trichina-infected guinea pigs to ascaris extracts. This sensitivity does not appear to be selec-

TABLE 10.—*Effect of whole extracts of various helminths and other tissues on guinea pigs sensitized to ascaris materials (A_1 and and A_s3).*

Shocking extract	Sensitizing extract	
	A_1	A_s3
A. lumbricoides	+++	+++
A. columnaris	++	+
T. leonina	+++	+++
P. maxillaris	+	–
M. expansa	–	–
F. magna	–	–
Macracanthorhyncus hirudinaceus	–	–
Guinea pig kidney	–	–
Group A erythrocytes	–	–

TABLE 11.—*Effect of ascaris and trichina extracts on guinea pigs infected with* T. spiralis.

Extract	No. of animals	Effect
Ascaris A_1	2	+++
A_2	2	+++
$A_1{:}8$	2	+++
A_3	2	+++
A_s3	2	+++
T. spiralis (ground larvae)	2	+++

tive for any particular chemical fraction of ascaris. Whole worm, deproteinized whole worm, (A_2) polysaccharide (A_3) and also fractions from which both protein and polysaccharide were removed (A_3 and A_7s) appeared to be effective. It will be observed from experiment 10 that ascaris-infected guinea pigs were not affected when injected with an emulsion of trichina larvae, while in experiment 11 it appeared that trichina-infected animals were affected by ascaris extracts.

DISCUSSION

From the reports of various investigators (see Sprent, 1949), it is evident that animals normally infected with ascaris worms, e.g., dogs, horses, cats, etc. are apparently highly susceptible to ascaris materials injected intravenously and, in some instances, by other routes of administration. It seems logical to assume that their susceptibility has arisen through infection. In the case of the guinea pig, it has been conclusively shown that small amounts (0.01 g) of all ascaris tissues and fluids are harmless in worm-free animals (Sprent, 1950), but that in ascaris-infected guinea pigs all whole tissue extracts are extremely toxic. There appears, then, to be no doubt that hypersensitivity may arise through infection. Anaphylactic shock has been produced in infected animals by whole and deproteinized extracts of adult worm tissues, and at least two fractions appear to be active. One is the fraction which is precipitated by alcohol from deproteinized extracts. It is readily soluble in water to form an opalescent solution, and it is not affected in its activity by phosphotungstic acid. The other is not precipitated by alcohol or by full saturation with ammoniun sulphate but is removed by phosphotungstic acid. Whether ascaris protein itself is involved in causing anaphylactic shock in infected animals is not known. These active fractions have been recovered from adult worm tissues, but they are not necessarily identical with the actual sensitizing agents occurring as a result of infection in the guinea pig, because the adult parasite does not mature in this animal. Whatever sensitizing agents occur during infection in the guinea pig, they must be derived from the larvae. There appear to be two possible sources for these substances, one is the excretory materials exuding from the larvae (metabolic products) and the other is the component materials of the larval body. With regard to the former, the results of experiment 6 showed that severe but not fatal anaphylaxis occurred following the injection into ascaris-infected guinea pigs of larval metabolic products as contained in the lungs of heavily infected animals. As to the latter source, whole larvae from the lungs of guinea pigs were evidently able to cause fatal anaphylactic shock in ascaris-infected animals (experiment 4). Accordingly, there appears to be no doubt that anaphylaxis-producing substances occur in the tissues, and probably also in the excretions, of the larvae.

It appears also that adult metabolic products contain a substance which will shock infected animals, and that this effect is not removed by trichloracetic acid. This material was not found to be antigenic in the sense of causing active sensitization (see experiment 7), but it might nevertheless contain a haptene which, although by itself able to produce shock, was not able to sensitize unless coupled to a larger molecule. Since animals sensitized against whole adult worm and against ascaris polysaccharide could be shocked with adult metabolic products (experiment 8), there is evidence for supposing that adult metabolic products contain substances which have chemical and immunological simi-

larities to substances occurring in the whole worm. Evidence to show that similar substances are present in the metabolic products of the larvae is lacking; no immunological relationship between adult tissues and larval metabolic products could be demonstrated (experiment 8), and, although the latter was able to shock infected guinea pigs, this effect was removed by precipitation with trichloracetic acid.

While it is evident that anaphylactic shock occurs in infected guinea pigs following the injection of adult tissues, adult metabolic products, larval tissue, and larval metabolic products, it has not been proved that these different materials owe their common property to identical substances.

As for the two fractions which were obtained from adult ascaris tissues and found to be active in causing anaphylaxis, it would not be possible to show their immunological significance without further knowledge of the chemical nature of the substances which cause sensitization in infected animals. The statement that two fractions are involved was made on the assumption that chemical separation was complete, an assumption which may with more precise technique, be proved wrong. It seems quite possible that the anaphylactic activity of fraction A_a3 which has been referred to as a polysaccharide may be due to the association with it of the same active substance responsible for the effect of extracts A_2s and A_3. This active substance may be so bound to the polysaccharide material that it is not removed from it by phosphotungstic acid, whereas when it is free, as it may be in extracts A_3 and A_2s, it is readily removed by phosphotungstic acid. This would explain why the polysaccharide fraction was less potent than extract A_2s (see experiment 4). Phosphotungstic acid was used by Campbell (1936) for removing nitrogenous material from ascaris polysaccharide; in the present experiments this reagent caused a reduction in nitrogen content of the polysaccharide fraction (A_a3) from 1.44 g percent to 0.47 g percent but did not remove its anaphylactic effect. Injection of 0.01 g of extract A_a3p caused fatal anaphylaxis, and this amount would contain 0.047 mg nitrogen. It is, therefore, not possible to state that the polysaccharide itself is active in causing anaphylaxis. As Campbell (1936) pointed out, it is not possible to eliminate the possibility of contamination until "chemical methods become as sensitive as biological reactions." The active substance occurring in extract A_2s and A_3 and possibly contaminating the polysaccharide fraction is probably the same or similar to the substance described by Rocha e Silva and Grana (1946) from deproteinized ascaris extracts. Sensitization to this substance probably occurs during the course of larval migration, because either through disintegration of some of the larvae or from their exudations, there are released into the tissues of the host related chemical substances, perhaps of a haptenic nature which becoming attached to larger molecules attain the properties of complete antigens. Hypersensitivity then occurs in the infected animal as a result of the antibodies which are produced. When substances with similar haptenic groupings, occurring in the various tissues and fluids of ascaris adults and larvae, are injected intravenously, anaphylaxis consequently results.

Cross-sensitization against other helminth tissues can only be explained fully when it can be demonstrated that sensitizing antigens with common immunological properties are actually released in the body of infected animals. The demonstration of common antigens

within the worm tissues is not sufficient, because while these may be present, they are not necessarily released during infection. Campbell (1936) isolated a polysaccharide from pig ascaris tissues which he demonstrated could sensitize guinea pigs. This polysaccharide he claimed (Campbell, 1937) was specific for *A. lumbricoides* from the pig, and by precipitation tests, he was able to distinguish it from a polysaccharide obtained from *A. lumbricoides* from man and from similar polysaccharides obtained from various other helminths. Oliver-González (1946) on the other hand, isolated a polysaccharide substance from *A. lumbricoides* from pigs which showed a high degree of cross reactivity in vitro with polysaccharides from other helminths belonging to the nematodes, cestodes and trematodes. His polysaccharide material evidently differed immunologically from that of Campbell in that as he points out, his method of extraction may have retained some common factor in all the different polysaccharides. This factor he believes is an isoagglutinogen-like material which may be destroyed by strong acid and alkali. However, the point at issue as far as cross-sensitization through infection is concerned is not what antigens occur in the worm, but what antigens come in contact with the host. Though the ascaris polysaccharides may have certain properties in common with other polysaccharides such as the Forssman polysaccharide, pneumococcus polysaccharides and group A isoagglutinogen (Oliver-González, 1946) there is evidence to show that these common immunological properties do not render ascaris-infected animals sensitive to these other polysaccharides (Sprent, 1950), and they did not produce anaphylaxis in guinea pigs sensitized to ascaris polysaccharide (experiment 10). The mode of preparation of the ascaris polysaccharide involved the use of trichloracetic acid which may have destroyed the common factor. Nevertheless, it appears evident from previous work (Sprent, 1950) and from experiment 9, that antigens common to certain other nematodes are released in ascaris-infected animals. The possibility that polysaccharides may be responsible for cross-sensitivity among nematode infections has been discussed previously (Sprent, 1950), and the present experiments have indicated that a fraction containing polysaccharide may be involved. However it appears possible that a contaminant, rather than the polysaccharide itself, is the effective agent.

SUMMARY

1. Guinea pigs infected with the larvae of *Ascaris lumbricoides* from pigs exhibited anaphylactic shock when injected intravenously with materials obtained from whole adult ascaris, isolated adult tissues, adult metabolic products, larval tissues and larval metabolic products.

2. A similar effect was produced by whole adult ascaris, isolated adult tissues and adult metabolic products, after treatment with trichloracetic acid.

3. Two chemical fractions active in producing anaphylaxis were obtained from deproteinized whole ascaris tissues. One was able to sensitize guinea pigs, was insoluble in alcohol and was unaffected in its effect by phosphotungstic acid. The other was unable to sensitize guinea pigs, was soluble in alcohol and was removed by phosphotungstic acid. A similar separation was effected by saturation of deproteinized ascaris tissues with ammonium sulphate.

4. Guinea pigs infected with decreasing doses of ascaris eggs showed that hypersensitivity through infection was

instigated by a dose of 150 eggs.

5. Immunological relationships were established by cross-sensitization between adult worm tissues, larval tissues and adult metabolic products.

6. Ascaris-infected guinea pigs were found to manifest anaphylactic shock when injected intravenously with extracts prepared from *Ascaris columnaris*, *Toxascaris leonina* and *Physaloptera maxillaris*, but not by extracts prepared from *Trichinella spiralis*, three cestodes and a trematode.

7. Guinea pigs sensitized with whole ascaris and with ascaris polysaccharide exhibited a similar cross-sensitization towards other helminth extracts as that exhibited by ascaris infected guinea pigs. No sensitization towards extracts of guinea pig kidney and human group A erythrocytes was manifested.

8. Guinea pigs infected with *T. spiralis* showed anaphylactic shock when injected with whole ascaris tissues, deproteinized ascaris tissues, ascaris polysaccharide and by ascaris tissue extracts from which both proteins and polysaccharide had been removed.

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RESPONSE OF THE SYRIAN HAMSTER TO ORAL ADMINISTRATION OF MODIFIED NEWCASTLE DISEASE VIRUS

REGINALD L. REAGAN, DOROTHY M. SCHENCK, H. ODETTE WERNER, AND A. L. BRUECKNER

From the Maryland State Board of Agriculture, Live Stock Sanitary Service Laboratory, University of Maryland, College Park, Maryland

The adaptation of the California strain (No. 11,914) of Newcastle disease virus to the Syrian hamster by intracerebral inoculation has been reported, as well as subsequent adaptations of modified virus to hamsters by intranasal, intratesticular, and intradermal routes of inoculation.[1–3] No attempts were made in this laboratory to establish Newcastle disease virus in hamsters by oral administration previous to the work to be reported herein.

TABLE 1.—*Hamster passages (oral) of hamster-modified Newcastle virus.*

Passage no.	No. inoculated	Hamsters paralyzed, moribund, or dead		Incubation period (in days)
		no.	%	
1	4	4	100	4-4-5-6
2	12	4	33.3	4-4-5-5
3	5	3	60	4-5-6
4	5	4	80	3-3-3-3
5	5	4	80	3-4-4-4
6	7	6	85.7	3-4-4-4-5-5
7	7	4	57.1	4-4-5-6
8	10	5	50	4-5-5-6-7
9	5	3	60	3-3-4
10	6	3	50	6-6-7
11	8	3	37.5	8-8-8

The modified hamster virus was first successfully established per os by utilizing 0.1 cc of a 10% virus-bearing hamster brain suspension of the 320th intracerebral passage as the initial inoculum. The virus of this passage titered 10^{-4} (table 2) intracerebrally in hamsters by the Reed and Muench[4] method of calculation and was verified to be Newcastle disease virus by a neutralization test using hamsters as the test host. Hamsters injected intracerebrally with this passage succumbed to Newcastle disease within 48 hours after inoculation. Four 4-week-old hamsters (average weight 30 g) were lightly anesthetized with ether, and the inoculum was placed on the posterior portion of the tongue. The hamsters developed signs of involuntary motor reactions, evidence of pharyngeal paralysis resulting in excessive salivation, and general paralysis in 4 to 8 days. These symptoms are similar to those occurring in hamsters infected by other routes. The brains of the paralyzed hamsters were removed aseptically, ground with alundum, and diluted to a 10% suspension with physiological saline. One-tenth cc of this suspension was placed on the posterior part of the tongue of the hamsters for the succeeding passage. Serial passage was continued by this method for 11 passages as shown in table 1. Upon intracerebral titration in hamsters, virus-bearing brain material from the 11th oral passage titered $10^{-4.5}$ (table 2). These passages by oral administration are being continued at the present time.

In order to determine the distribution of Newcastle disease virus in the affected hamster, the heart, liver, spleen, kidneys, lungs, feces, spinal cord, and brain of hamsters of the 11th passage were removed separately with aseptic precautions, ground with alundum, and diluted to 10% suspensions with physiological saline. Because of possible bacterial contamination, suspensions of the

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lungs, kidneys, liver, spleen, and feces were treated with streptomycin in the ratio of 1 mg per cc of suspension and with penicillin in the ratio of 500 units per cc of suspension before embryonated egg and hamster inoculation. One-tenth cc of each suspension was injected into the allantoic sac of each of six 7-day embryonated White Leghorn eggs and into each of four 4-week-old Syrian hamsters by the intracerebral route of injection. The results of these attempted isolations are given in table 3. Only the brain tissue yielded virus.

TABLE 2.—*Comparative titration test results.*

Hamster passage	Virus dilutions					Titer
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
320th intracerebral	4/4	4/4	4/4	2/4	0/4	10^{-4}
11th oral			4/4	4/4	0/4	$10^{-4.5}$

Numerator of fraction denotes number of deaths; denominator denotes number of hamsters inoculated.

TABLE 3.—*Distribution of Newcastle virus in hamster tissues.*

Tissue suspension	Test host	
	Embryonated eggs (allantoic route of injection)	Hamsters (intracerebral route of injection)
Blood	−	−
Liver	−	−
Spleen	−	−
Kidneys	−	−
Lungs	−	−
Feces	−	−
Spinal cord	−	−
Brain	+	+

− No evidence of virus; + virus present.

A neutralization test was conducted in 9-day embryonated eggs using allantoic fluid harvested from those eggs infected by the brain suspension of the 11th oral hamster passage. A second neutralization test was performed using hamsters as the test host and virus-bearing brain material from the 11th oral passage. In both tests the virus was completely neutralized by Newcastle-immune chicken serums but was unaffected by normal chicken serums.

In order to eliminate the possibility that the virus suspension was being aspirated into the lungs or carried up into the nasal passage, the first three passages were repeated using the following technique of inoculation: a catheter was passed down the esophagus into the stomach and the inoculum introduced through the catheter. From 50% to 83% of the hamsters succumbed to the disease on the 4th or 5th day post inoculation and exhibited symptoms similar to those described above. A neutralization test in hamsters using brain suspension of the 3rd stomach passage was positive for Newcastle disease virus.

SUMMARY

Upon trial of the 320th intracerebral hamster passage, NDV, California strain No. 11,914, hamsters were infected by oral administration of the virus-bearing brain material. No attempts had been made with hamster modified virus of intracerebral passages previous to the 320th. Oral administration was continued through 11 serial passages.

Virus was isolated in 7-day embryonated eggs using hamster brain suspension from the 11th oral passage. Neutralization tests with this infected allantoic fluid and with brain suspension from the 11th passage confirmed the virus to be that of Newcastle disease.

Hamsters infected intracerebrally, intranasally, intradermally, intratesticularly, and orally show similar symptoms such as involuntary motor reactions, excessive salivation from pharyngeal paralysis, general paralysis, prostration, and death.

A SEARCH FOR POLIOMYELITIS VIRUS IN HOUSE DUST AND A STUDY OF THE EFFECTS OF DRYING ON THE VIABILITY OF THE VIRUS

HAROLD K. FABER, LUTHER DONG, AND ROSALIE J. SILVERBERG

From the Department of Pediatrics, Stanford University School of Medicine, San Francisco 15, California

Since 1910 it has been suggested from time to time[1-3] that poliomyelitis might be transmitted by house dust, and in 1944 we reported[4] experiments indicating that infection might be conveyed through the air in dry droplet "nuclei." These possibilities imply that the virus withstands the lethal effects of drying for a certain period of time. Direct evidence of its presence in dust and of its resistance to drying is, however, scanty. There is only one recorded instance in which it was recovered from house dust; this is the report by Neustaedter and Thro[5] in 1911 of a positive test with sweepings from the sickroom of a patient. There are only a few studies, all of them made 40 years ago, on desiccation in vitro, and the results of these are both conflicting and inconclusive. The subject, therefore, seems to us to be of sufficient importance to warrant further exploration.

Flexner and Lewis[6] in 1910 found that infected cord dried over caustic potash remained active for 7 days. Levaditi and Landsteiner[7] in the same year stated that virus dried over phosphorus pentoxide was virulent for at least 24 days. Römer and Joseph,[8] also in 1910, found that infected cord dried in vacuo was still active at 28 days. On the other hand, Leiner and von Wiesner[9] in the same year, found that emulsions spread in thin layers and dried at 37 C for 4 hours or at room temperature for 24 hours were no longer active. These authors pointed out that the discrepancy between their tests and those of the other investigators was probably due to greater completeness of drying in their own experiments.

In 1940, we attempted to inactivate MVA strain* by desiccation and prolonged storage for the production of an immunizing antigen. The original material was not titrated for infectivity. The cords of 4 paralyzed rhesus monkeys dying or sacrificed 1, 1, 3 and 1 days after the onset of symptoms were made up into individual 10% saline suspensions, designated in the table as a, b, c and d respectively. Two ml samples of each suspension reduced to apparent dryness from the frozen state in a lyophile apparatus and stored in vacuo

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* A mixture, made up in the early 30's by Dr. E. W. Schultz of Stanford University, of the Rockefeller MV ("mixed virus") strain and another strain (A) obtained from Dr. Charles Armstrong.

at 4 C were reconstituted at varying intervals with distilled water at 20% concentration of original tissue and tested by intracerebral inoculation of 1.0 ml, each test on a single rhesus monkey. The results, which have not been previously published, are shown in table 1.

TABLE 1.—*Effect of lyophilization and storage in vacuo on poliomyelitis virus (MVA).*

Suspension	Interval after drying	Poliomyelitis	Incubation period
a	4½ mo.	+	13 days
a	7 "	0	
a	7½ "	0	
b	7½ "	+	7 days
c	7½ "	0	
d	7½ "	0	

The two positive results in this preliminary study, indicating that desiccated poliomyelitis virus might remain viable for fairly long periods, at least in vacuo at 4 C, led us to further investigations, including the present ones. However, in referring to our original notes, we find that the lyophile apparatus had not been working perfectly and that repeated thawing of the material occurred during the drying process, which casts some doubt on the completeness of drying attained.

In our subsequent experiments with inhalation, 4 monkeys, their heads placed in a closed chamber, were made to inhale air contaminated with finely atomized virus suspensions (OH and Per strains), the droplets being found to be almost instantaneously dried. Of 10 cynomolgus monkeys with olfactory blockade by zinc sulphate, 4 developed paralytic disease by nonolfactory entry, while of 7 without blockade, 6 developed the paralytic disease with olfactory entry. It was thus proved that infection by inhalation of dry droplet nuclei was possible under the conditions of the tests, which were considered analogous in some respects to human conditions of exposure.

Our present investigations fall into two categories: search for virus in house dust from homes in which one or more recent cases of poliomyelitis had occurred; and a study of the effect of desiccation on the viability of virus suspended in aqueous mediums, in oronasopharyngeal secretions and in feces; the last two because they are the natural mediums in which poliomyelitis virus is eliminated from the body, and because their organic components might conceivably form protective matrices against rapid inactivation by desiccation and other adverse influences.

HOUSE DUST

Methods.—During 1943, 1944, and 1945, when poliomyelitis was prevalent in San Francisco and its environs,* we collected dust from 118 residences in which acute cases of the disease had occurred within 2 months, except in 2 instances. Some samples were collected by sweeping or dusting in the sickroom and others from the household vacuum cleaner. All were kept at 4 C until extracted for testing, for which we used the method of high-speed centrifugation (18,000 r.p.m. for 4 hours) developed in this laboratory.[10] Individual samples ranged in weight from 0.25 to 50 g, two-thirds of them weighing 15 to 30 g. They were tested singly in some instances and in others, in pools of 2 to 7 individual specimens by intracerebral inoculation into 44 rhesus or 14 African vervet monkeys. Careful histological inspection of the CNS was made in all cases for signs of inapparent infection. Because of a shortage of monkeys, only a minority of the specimens could be tested within two months of the onset of the disease and with most of the remainder, inoculation had to be delayed for 12 to 28 months after onset. One sample was collected 7 days after onset and inoculated singly. Table 2 shows the periods in-

* In San Francisco the mean noon relative humidities (31 year period) for July, August and September are 70%, 71% and 64%, respectively; and the mean noon temperatures (68 year period) are 58.9 F (14.9 C), 59.4 F (15.2 C) and 61.5 F (16.4 C) for the same months (data from U. S. Weather Bureau). There is little year to year variation. These conditions are relatively favorable to drying, especially during September. About two-thirds of our dust samples were collected during these three months, and nearly half of them during September.

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tervening between the beginning of symptoms and the collection of dust, and inoculation. It will be noted that 33 specimens were collected within 10 days and 110 within one month, while 18 specimens were inoculated within one month and 38 within two months after onset.

Results.—No poliomyelitis virus was detected in any of the samples.

DESICCATION OF VIRUS IN VITRO

The Lansing virus used was a mouse-adapted strain obtained originally from Dr. Charles Armstrong of the National Institute of Health. Two stock suspensions were employed, each with a PD_{50} of 3.9 as first prepared and as retested once for each during the course of the experiments. Wis'45, a highly virulent human strain originally obtained from Dr. Paul F. Clark of the University of Wisconsin, had a PD_{50} of 4.5 for the stock suspension used. With this strain the test animals were cynomolgus (M. irus) monkeys. For tests of oronasopharyngeal washings and of stools for virus, the materials were centrifuged either at 18,000 r.p.m. for 4 hours, or at 30,000 r.p.m. for 1 to 1½ hours, and the pellets resuspended in saline. The amounts of intracerebral inoculums were 0.03 ml for mice and 0.8 ml for monkeys. Our criteria for positive infections in mice were typical paralysis, or typical lesions in brain and cord in animals found dead. In the case of inoculated monkeys, central nervous tissues were always examined microscopically for both positive and negative confirmation.

TABLE 2.—*Tests for virus in dust from dwellings of 118 poliomyelitis patients.*

Collection, days after onset:	0–10	11–20	21–30	31–40	41–60	>60	
Inoculation, time after onset							Total samples
Days							
0 to 10	1	0	0	0	0	0	1
11 to 20	7	0	0	0	0	0	7
21 to 30	3	7	0	0	0	0	10
31 to 40	1	3	4	0	0	0	8
41 to 60	0	6	5	1	0	0	12
Months							
2 to 3	0	0	0	1	0	0	1
12 to 17	1	1	0	0	0	0	2
18 to 28	20	31	20	1	3	2	77
Total samples	33	48	29	3	3	2	118

A. Aqueous suspensions (Table 3)

1. Drying in vacuo from the frozen state: two experiments.—Two 10% aqueous suspensions of Lansing, clarified by brief high-speed centrifugation, were frozen and then dried in a vacuum chamber for 2½ and 5 hours, respectively. From the first (exp. III-1), aliquots were reconstituted in volumes of saline equivalent to the original volume immediately at the end of the drying process and after 1, 3, 6 and 10 days of storage in air at room temperature. All tests were negative excepting a single animal (1/8*) inoculated with the 3-day sample at a dilution of 1:150 of original virus tissue. From the second (exp. III-2), tested immediately after drying and reconstitution 2/10 mice at 1:10 and 1/10 at 1:20 dilutions developed poliomyelitis.

2. Drying at atmospheric pressure at room temperature: 5 experiments.—In the first experiment (III-3), 0.5 ml of clarified 10% Lansing suspension was spread in a thin film in a petri dish and stored in the dark for 46 hours. Drying appeared complete at 24 hours. It was reconstituted in saline at a dilution of 1:20 of original tissue, from which 1/10 of the inoculated mice developed poliomyelitis. In the next 3 experiments (III-4, -5, -6), 10% aqueous Lansing suspensions in amounts of 0.2 to 1.0 ml were dried over calcium chloride in a desiccator at room temperature for 16 to 17 hours. All tests were negative with

* Numerator: number of animals developing polio; denominator: number of animals inoculated.

one exception (III-4, 1.0 ml) in which 3/8 of the mice inoculated with 1:20-dilution developed poliomyelitis. In the 5th experiment (III-8), 1.0 ml of suspension of the Wis'45 strain, dried for 16 hours, reconstituted in saline, diluted to 1:12 of original tissue and inoculated intrathalamically into two cynomolgus monkeys gave negative results as shown by absence of symptoms and of lesions in brainstem and cord.

B. Suspensions in oronasopharyngeal secretions: 5 experiments (Table 4)

Secretions were obtained by washing the oronasopharyngeal surfaces of normal cynomolgus monkeys with distilled water at frequent intervals for about 1½ hours. The same water, 30 ml originally, was used throughout. Aliquots were mixed with equal amounts of the stock suspensions; 10% Lansing, 42% Wis'45. The mixtures were spread in thin films in petri dishes or on glass slides and dried in a desiccator over calcium chloride at room temperature and atmospheric pressure for 4 to 16 hours. Experiments IV-1, IV-2, IV-3 and IV-5 were run simultaneously with, and under the same conditions as III-4, III-5, III-6 and III-8, but with separate wet controls (virus+washings). It should be noted that the results of these parallel tests with aqueous suspensions and suspensions in washings, were essentially identical. A small amount of residual viral activity was present in experiment IV-4 in which the drying

TABLE 3.—*Effect of drying on viability of poliomyelitis virus (Lansing, Wis'45): aqueous suspensions.*

Exp. no.	Strain	PD_{50} orig. susp.	Volume dried (ml)	V/A	Temp.	Time	Results				
							Dried		Wet Control		
							Lowest dilution tested	Higher dilutions	Dilution tested		PD_{50}
III-1*	Lansing	3.9	0.50	V	0	2½ hrs.	1:500 0/8	0			3.9
						1 day	1:500 0/8	0			
						3 days	1:150 1/8	0			
						6 "	1:50 0/8	0			
						10 "	1:20 0/8	0			
III-2	Lansing	3.9	1.00	V	0	5 hrs.	1:10 2/10	1:20 1/10			
								>1:20 0			
III-3	Lansing	3.9	0.50	A	±21	46 "	1:20 1/10				
III-4	Lansing	3.9	1.00	A	±21	16 "	1:20 3/8		1:20	5/6	
III-5	Lansing	3.9	0.20	A	±21	16 "	1:100 0/6		1:100	5/6	
III-6	Lansing	3.9	0.50	A	±21	17 "	1:40 0/7	0			3.9
III-7*	Lansing	3.9	0.25	V	±21	¼ "	1:40 0/10		1:40	8/10	
						1 "	1:40 0/10				
						2 "	1:40 0/10				
						3 "	1:40 0/10				
						4 "	1:40 0/10				
						5 "	1:40 0/10				
						6 "	1:40 0/10				
			0.25	A	±21	4½ "	1:40 0/10				
III-8	Wis '45	4.5	1.00	A	±21	15 "	1:12 0/2				

V/A: dried in air or in vacuo. Specimens dried in air for 4½, 16 and 17 hrs. were over calcium chloride in a desiccator.
Time: total time elapsed after drying started.
Dilution: referred to concentration of original tissue.
Numerator: number of animals developing polio. Denominator: number of animals inoculated.
* Specimens removed from the vacuum chamber at 2½ hours in exp. III-1 and at ¼ hour in exp. III-7; then kept in air at room temperature (over calcium chloride in exp. III-7), and reconstituted in saline at stated interval, as measured from the commencement of drying in vacuo.

3. Drying in vacuo at room temperature: 1 experiment.—In this experiment (III-7), by using only 0.25 ml of suspension spread out in a very thin film on glass slides, it was possible to effect complete drying in a vacuum desiccator in 30 minutes. Samples reconstituted in saline to a dilution of 1:40 of original virus tissue at ½, 1, 2, 3, 4, 5 and 6 hours after drying was begun were uniformly negative. A similar preparation dried at atmospheric pressure for 4½ hours was also negative. We regard this experiment as the most satisfactory of the series in respect to certainty of drying.

period was 4 hours; no parallel experiment with aqueous suspensions was made in this instance.

No protective effect of oronasopharyngeal secretions against the lethal action of drying on poliomyelitis virus is apparent in these experiments.

petri dish and dried for 16 hours at room temperature and atmospheric pressure over calcium chloride, but not triturated during the process. We are not sure that the mixture was completely dried. At 16 hours the material tested at a dilution of 1:12 of original virus tissue was

TABLE 4.—*Effect of drying on viability of poliomyelitis virus (Lansing, Wis'45): suspensions in oronasopharyngeal washings.*

Exp. no.	Strain	PD_{50} orig. susp.	Volume dried (ml)	Time	Results			
					Dried		Wet controls	
					Lowest dilution tested	Higher dilutions	Dilutions tested	PD_{50} if titrated
IV-1	Lansing	3.9	1.0	16 hrs.	1:20 3/6		1:20 6/6	
IV-2	Lansing	3.9	0.2	16 "	1:100 0/6		1:100 5/6	
IV-3	Lansing	3.9	0.5	17 ".	1:40 0/7	1:80 1/7	1:40 5/7	
						>1:80 0	1:320 6/7	
IV-4	Lansing	3.9	0.5	4 "	1:40 2/10	1:120 2/10		3.8
IV-5	Wis'45	4.5	1.0	16 "	1:12 0/2		1:12 2/2	
							1:1000 0/2	

All specimens were dried over calcium chloride in a desiccator at ±21 C at atmospheric pressure.
Time: hours elapsed after drying was started.
Numerator: number of animals developing polio. Denominator: number of animals inoculated (mice for the Lansing strain, monkeys for the Wis'45 strain).

TABLE 5.—*Effect of drying on viability of poliomyelitis virus in stools (Wis'45, Gib).*

Exp. no.	Strain	PD_{50} original suspension	Weight dried (g)	V/A	Temp. (C)	Time. (hrs.)	Dilution* or weight tested	Results	
								Dried	Wet control
V-1	Wis'45	4.5	1.0	A	±21	16	1:12	2/2	
V-2	Wis'45 (naturally excreted)		20	V	±21	20	4.0 g.	0/2	1/1
							0.4 g.		0/2
V-3	Gib (in patient's stool)		50	V	±21	22	2.3 g.	0/2	2/2
							0.23 g.		2/2
							0.023 g.		1/2

V/A: dried in vacuo or in air over calcium chloride in a desiccator.
Time: hours elapsed after drying was started.
Numerator: number of monkeys developing polio. Denominator: number of monkeys inoculated.
* Referred to original tissue or weight of stool.

C. Mixtures with stool: 3 experiments (Table 5)

Mixtures of normal cynomolgus stool with the Lansing virus were unsatisfactory for our purposes, since the wet controls showed only the slightest or no remaining viral activity. The nature of the inactivating substance is unknown. Wis'45 was, however, recoverable at dilutions of at least 1:1000 in the wet controls. In experiment V-1, 1.0 g of freshly passed normal cynomolgus stool was mixed with 0.5 ml of 42% suspension of Wis'45 virus, spread out in a

found to be active, both subinoculated monkeys coming down. In experiments V-2 and V-3, under otherwise similar conditions, the material was repeatedly triturated during the process and reduced to a fine dust. In V-2, we used naturally excreted virus contained in the stools of a cynomolgus monkey paralyzed after intravenous inoculation of large amounts (>24,000 PD_{50}) of Wis'45. In experiment V-3 we used stools, known to be positive, of a patient (Gib) with poliomyelitis. The results of both V-2 and V-3 were negative

after drying, the wet controls being positive.

In this small series, inactivation by drying occurred in the two experiments where drying was probably complete. Neither monkey nor human stool appeared to protect virus from inactivation.

DISCUSSION

The tests of house dust indicate that any poliomyelitis virus that may be eliminated by patients and become incorporated in dust fails to survive for periods of about two weeks or more. While it cannot be said from these tests alone that activity cannot survive for shorter periods, the experiments on desiccation indicate that with adequate drying, such as might be expected with air-borne infective particles under ordinary indoor conditions of temperature and humidity leading to dust formation, the period of residual viral activity is much briefer.

From our in vitro experiments, it is evident that desiccation exerts a prompt and marked, if apparently somewhat variable, inactivating effect on poliomyelitis virus. On the other hand, even small amounts of residual moisture may prolong its survival. Incomplete drying may possibly account for the few instances in our study in which inactivation was incomplete. This is suggested by the fact that complete inactivation occurred in all instances where the amount of material subjected to drying was small (less than 0.5 ml or g) and where, with larger amounts, special care was taken to reduce the material, during drying, to fine powder.

The rapidity with which inactivation can follow drying is strikingly shown in experiment III-7, where the material reconstituted half an hour after drying was started showed no activity. Neither temperature, exposure to air (oxygen) nor light appeared to be important inactivating factors in our experiments, since inactivation occurred at freezing as well as at room temperatures; in vacuo as well as at ordinary atmospheric pressure; and all specimens were kept under thick glass, for most of the time in the dark.

The fact that oronasopharyngeal mucus exerted little if any protective effect against inactivation by drying in our experiments is of considerable practical interest. It has been shown by Wells[11] that droplets expelled from the nose and mouth, if of small size, are rapidly dried in the air ("droplet nuclei") and may be important instrumentalities in the spread of such air-borne viral infections as influenza and measles. In our previous experiments,[4] already cited, on inhalation in which infection was readily induced with droplet nuclei and from which we concluded that air-borne contagion of poliomyelitis deserved further consideration, it must be pointed out that virus was inhaled within a very brief period, probably not more than a few seconds, after its dispersal into the air. Our present study suggests that poliomyelitis virus contained in such fine dry particles might remain viable less than half an hour.

The observations on stools, though few in number, also indicate that desiccation of fecal matter inactivates the incorporated virus and also suggest that residual moisture permits its continued viability. Further observations on these points may be desirable.

Our observations on house dust and drying may be correlated with certain epidemiological aspects of the human disease. It has been shown, notably by Casey and his associates,[12] by Pearson and Rendtorff[13] and by Francis and

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Brown[14] that poliomyelitis is spread mainly by "contact," a term which, in addition to actual physical contact, should include exposure to droplets expelled from nose or mouth and to fecal discharges contaminating fomites, at close quarters or within brief periods after elimination. As between nasal/oral and fecal transfer of infection, the period of maximum infectivity[15] favors the former since it corresponds more closely with the time, a few days before[16] to about a week after[17] the onset of symptoms, when virus is detectable in the pharynx, than with the considerably longer time after onset when it is present in the stools.[18]

The rarity of cross-infections in hospitals housing patients with poliomyelitis and of epidemics traceable to exposure in schools, movie theaters, etc. is in harmony with our present experiments and suggests that the disease is not as a rule spread through the air by dry droplet nuclei, contrasting in this respect with some air-borne diseases such as measles and influenza. We therefore suggest that poliomyelitis virus is most dangerous and is transmitted most readily while it is in the moist state; as by inhalation at close quarters of large wet droplets, by direct physical contact (lips, hands), by ingestion of contaminated food and drink, and by the use of contaminated eating and drinking utensils before drying. Various situations in the family bathroom and toilet can be readily envisioned in which transfer of infected oral and fecal material in the moist state could readily occur.

The importance of reservoirs of moist virus, such as open privies, from which flies may convey virus to considerable distances, appears to be less than was formerly believed,[19] but cannot be wholly discounted.

House dust is probably unimportant in the spread of poliomyelitic infection.

SUMMARY

1. One hundred and eighteen samples of house dust from dwellings of patients with acute poliomyelitis were tested for poliomyelitis virus, 38 within 60 days after onset. All tests were negative.

2. Mixtures of poliomyelitis virus (mouse-adapted Lansing and human strains) with water, with oronasopharyngeal washings from normal cynomolgus monkeys, and with stools were subjected to drying under various conditions of temperature and atmospheric pressure and for periods ranging from one-half hour to 46 hours. With maximal desiccation, inactivation of the virus was prompt and complete. On the other hand, even small amounts of residual moisture appeared to favor continuing activity.

3. Neither oronasopharyngeal mucus nor stool appeared to protect the virus against the lethal effects of drying. In these experiments, the latter occurred independently of variations in temperature and of exposure to oxygen and light.

4. It is suggested that our observations are related to the conditions under which human poliomyelitic infection is transmitted, and favor the concept of intimate contact as against air-borne spread by dry droplet nuclei or by house dust.

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LYSIS OF *PASTEURELLA PSEUDOTUBERCULOSIS* BY BACTERIOPHAGE

J. B. GUNNISON, M. C. SHEVKY, V. K. ZION, AND M. J. ABBOTT
From the Division of Bacteriology, University of California, School of Medicine, San Francisco 22, California

A bacteriophage for *Pasteurella pestis*, also able to lyse 31 of 40 strains of *Pasteurella pseudotuberculosis*, was adapted to a strain of *P. pseudotuberculosis* and subsequently lysed all 40 strains of this species (Lazarus and Gunnison, 1947; Gunnison and Lazarus, 1948). This report deals with the effects of temperature of incubation on lysis of *P. pseudotuberculosis* and with the relationships between its sensitivity to lysis and its morphologic and antigenic properties.

MATERIALS AND METHODS

Bacteriophages.—The purified phage, designated as "P phage," described by Lazarus and Gunnison (1947) was used. This phage, isolated by Advier (1933), has been transferred repeatedly on an avirulent strain of *P. pestis* (A 1122). P phage was propagated repeatedly on strains of *P. pseudotuberculosis* (Spokane and 40) against which it originally had little action; these adapted phages are designated as "PTB phage." Likewise the P phage was propagated on strains of *P. pseudotuberculosis* (NIH B111 and 39) against which it originally was highly active. Phages were transferred by adding 0.5 ml from the previous passage to 10 ml of a two hour broth culture of the bacteria and incubating at 37 C until clear.

Cultures.—To the 27 strains of *P. pseudotuberculosis* previously listed (Lazarus and Gunnison, 1947) have been added 2 strains from the National Collection of Type Cultures, 9 from the American Type Culture Collection, and 2 recently isolated from a canary (39) and a chinchilla (40) at The Hooper Foundation for Medical Research.

Mediums.—The broth medium contained proteose peptone #3 (Difco), 20 g; glucose, 0.5 g; sodium chloride, 5 g; disodium phosphate, 5 g; distilled water, 1000 ml (pH 7.3). For a solid medium 15 g of agar per liter were added to this broth. For a semisolid medium used in motility tests 1.5 to 1.75 g of agar per liter were added to the broth.

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Phage tests.—Bacterial cultures, grown in broth at either 37 C or at room temperature (18 to 20 C) for 18 to 24 hours, were spread with a loop having an inside diameter of 3 mm, over circular areas 2 cm in diameter on the surface of dry agar plates. As soon as the inoculum had dried, a loopful of phage was touched to the center of each area, taking care not to disturb the film of organisms. The plates were inverted after a few minutes and held either at 37 C or at room temperture (18 to 20 C) for 48 hours.

Motility tests.—A droplet of culture was mixed with a droplet of 0.02% aqueous methylene blue and examined microscopically under a cover slip. For tests in semisolid agar, the medium was poured into Petri dishes (75 mm in diameter) having lids lined with filter paper to absorb moisture as the dishes could not be inverted. A loopful of culture was touched lightly to the surface of the medium at 4 separated points. The cultures were kept at room temperature and observed frequently.

Stains.—For demonstration of flagella, Leifson's stain (Leifson, 1930) was most satisfactory. Cultures suspended in distilled water to give a faint turbidity either were allowed to stand for 15 minutes, or were centrifuged and resuspended in distilled water. After placing on slides, the preparations were steamed gently for 7 minutes over boiling water.

To demonstrate material outside the cell wall the india ink technique and the methods of Hiss (1905), Maneval (1929), Knaysi (1941), Jasmin (1945), and Kleineberger-Nobel (1948) were used.

Agglutination tests.—Antiserums were prepared by repeated intravenous injection of rabbits with suspensions of formalin-killed pasteurellas. Antigens were prepared from agar cultures of *P. pseudotuberculosis* grown at either 37 C or at room temperature, suspended in 0.3% sodium chloride solution, and heated for 30 minutes at either 60 C or at 100 C. The suspensions were shaken vigorously with glass beads for 30 minutes and filtered through cotton. Macroscopic agglutination tests in 13×100 mm tubes were incubated at 37 C overnight.

RESULTS

Lysis by phage.—At the conclusion of previous experiments, the 40 cultures of

P. pseudotuberculosis were stored at 10 C on agar slants under oil for 6 months. Upon retesting for lysis after storage, it was found that 16 strains originally lysed by P phage at 37 C had lost this sensitivity (table 1). It was noted that the results of tests left accidentally at 20 C differed from those incubated at 37 C. Accordingly the effect of repeated transfer at 37 C or at 20 C on sensitivity to lysis was tested. Daily subcultures of each strain were made in broth and incubated at the respective temperatures. After 10 such transfers, the cultures were retested with the results shown in table 1.

TABLE 1.—*Lysis of* P. pseudotuberculosis *by P and PTB phages at 37C and at 20 C.*

P. pseudotuberculosis cultures	Tested at	Number of strains lysed of 40 tested	
		by P phage	by PTB phage
Before storage	37 C	31	40
After 6 months storage at 10 C	37 C	15	40
After 10 subcultures at 37 C	37 C	26	40
After 6 months storage at 10 C	20 C	8	20
After 10 subcultures at 20 C	20 C	8	20

P phage = phage propagated on *P. pestis* (avirulent strain A 1122).
PTB phage = P phage adapted to *P. pseudotuberculosis* (Spokane strain).

The number of subcultures at 37 C required to restore sensitivity to lysis by P phage, lost during storage, varied with different strains; some regained susceptibility after a single transfer, others were still resistant after 10 transfers. Two of the strains lysed by P phage at 20 C were transferred 30 times at this temperature without loss of sensitivity to PTB phage but with gradual decrease in sensitivity to P phage.

Cultures of *P. pseudotuberculosis* repeatedly transferred in broth at 37 C sometimes retained their sensitivity to lysis when tested at 20 C but usually could not be lysed after one or two subcultures at 20 C. Cultures grown in broth at 20 C and tested at 37 C usually behaved as if they had been grown at 37 C. Subcultures of bacteria resistant to phage at 20 C were just as sensitive to lysis at 37 C as were the parent cultures. Consequently, the differences in sensitivity to lysis at the two temperatures cannot be explained by the development of resistant mutants during incubation at the lower temperature.

Based on their reactions with P and PTB phages, following repeated subculture at 37 C and 20 C respectively, the 40 strains of *P. pseudotuberculosis*, fell into four categories (table 2).

TABLE 2.—*Lysis of 40 strains of* P. pseudotuberculosis *by P and PTB phages at 37 C and 20 C after repeated subculture at the respective temperatures.*

Number of strains in category	Lysis			
	P phage		PTB phage	
	37 C	20 C	37 C	20 C
14	−	−	+++	−
6	++	−	+++	−
12	++	−	+++	+
8	+++	++	+++	++
Control *P. pestis* (A 1122)	+++	+++	+++	+++

− = no complete lysis.
+ = complete lysis with undiluted phage.
++ = complete lysis with phage diluted 10^{-1} to 10^{-3}.
+++ = complete lysis with phage diluted 10^{-4} to 10^{-6}.

P. pestis (A 1122) was lysed by high dilutions of both P phage and PTB phage at 20 C and 37 C (table 2). Repeated subculture at either temperature did not alter the high sensitivity of this organism.

Eight strains of *P. pseudotuberculosis* behaved throughout like *P. pestis* rather than like members of their own species, except that they were not lysed by as high dilutions of either PTB or P phage at 20 C as was *P. pestis* itself (table 2). P phage was repeatedly propagated on two of these strains (NIH B 111 and 39). The resulting lysates behaved

exactly like the original phage; i.e., the phage did not become adapted but retained the properties of the P phage.

From strains representative of the four categories, 10 to 40 colonies were isolated and tested with phage. All colonies from those strains originally sensitive to P phage were lysed. From a strain (Spokane), originally not lysed by P phage, approximately half the isolated colonies were lysed and among these many were unstable; i.e., they were lysed if tested immediately ,but became resistant on standing at 20 C for 24 hours. These unstable colonies were restored to sensitivity by growth at 37 C for 24 hours. From resistant and sensitive colonies of the Spokane strain, 93 daughter colonies were isolated; the sensitive parent colonies tended to breed true, but resistant parent colonies produced a few sensitive daughter colonies. The sensitivity to phage action of a given strain at a given moment apparently depends in part upon the proportion of cells susceptible to lysis under the conditions of the test.

Morphology.—In agreement with Arkwright (1927) and Weitzenberg (1935), it was noted that *P. pseudotuberculosis* was usually motile when grown at about 20 C but rarely at 37 C. The proportion of motile cells, which was low with some strains, was increased by daily transfer in broth. Selections from the hazy margins of rapidly spreading growth on semisolid agar showed a high number of actively motile cells with all strains tested.

Using Leifson's stain, cells of different strains revealed flagella of varying number and position. Small coccoid or ovoid cells and short rods often presented a single flagellum or two flagella in subterminal bipolar position; larger cells frequently showed 5 to 7 flagella and sometimes as many as 16. Often the flagella were so intertwined that the number could not be determined. In some strains the cells appeared to be surrounded by pale pink, amorphous material, possibly a "slime layer," from which extended flagella-like structures, suggestive of those described by Pijper (1938) using darkfield illumination of living cells. Attempts to stain this possible slime layer by other methods were not entirely successful, but it seemed to be present on occasional cells of all strains whether grown at 20 C or 37 C. Flagella and the apparent slime layer were seen both on strains lysed by phage and those not lysed.

The colonial morphology was noted whenever colonies were selected for lytic tests. Most strains produced rough, smooth, and intermediate colonies with smooth forms predominating at 20 C and rough forms at 37 C. Conceivably the absence of a slime layer, rather than changes within the cells, might account for roughness. Lysis was more often associated with rough colonies than with smooth ones, but the correlation was not complete.

Serologic tests.—Antigens of all strains of *P. pseudotuberculosis* tended to agglutinate spontaneously in 0.85% sodium chloride especially when grown at 37 C, and some were also unstable in the 0.3% solution used in these tests. Suspensions stable in 0.3% salt solution sometimes were clumped in the presence of low dilutions of serum from presumably normal rabbits. Suspensions washed twice in 0.3% salt solution were usually more likely to show spontaneous clumping than were unwashed suspensions, perhaps because the removal of surface material from the cells exposed the rough antigen and rendered them more unstable. Soluble antigen was demonstrated in the supernatant washings from such suspensions by precipitation tests with *P. pestis* antiserum.

Strains which agglutinated spontaneously tended to be more sensitive to lysis by P phage and to contain a higher proportion of rough cells than those not so agglutinated, suggesting a relationship between roughness and sensitivity to P phage. It seems possible that the smooth surface antigen or perhaps the slime layer may mask the components of the cell sensitive to phage and thus protect the cell from lysis.

Schutze (1928), Kauffman (1932), and Bhatnagar (1940) have shown that *P. pseudotuberculosis* possesses the following antigenic components: (a) a somatic antigen common to all strains and shared with *P. pestis;* (b) a group specific somatic antigen dividing the species into 4 groups; (c) a type specific somatic antigen dividing groups I and II each into two types; and (d) a flagellar antigen, produced at 20 C but not at 37 C, common to all strains. These findings have been confirmed in the present study and an attempt has been made to determine the relationship of these antigenic factors to susceptibility to lysis.

All strains from which stable suspensions could be prepared after growth at 37 C were agglutinated by *P. pestis* antiserum. When grown at 20 C, approximately half the strains were not agglutinated by *P. pestis* antiserum and the remainder reacted to a lower titer than when grown at 37 C. Hence, at 20 C some strains either failed to produce the antigen shared with *P. pestis* or this antigen was masked by another surface antigen. Cultures transferred 10 times at 37 C still agglutinated to high titer with *P. pestis* antiserum, but those transferred at 20 C tended to become less agglutinable.

Strains resistant to lysis by P phage at 37 C agglutinated to as high titer, when grown at 37 C, as did susceptible ones. At 20 C, all strains lysed by P phage were agglutinated by *P. pestis* antiserum, but a number of strains which were not lysed were agglutinated also. Moreover, antigens from resistant secondary growth reacted as readily with *P. pestis* antiserum as did the sensitive parent cultures. Hence, the presence of an antigenic component shared with *P. pestis* did not necessarily mean that the organisms would be lysed by P phage.

In some strains the antigen common to *P. pseudotuberculosis* and *P. pestis* was heat stable; it still reacted with *P. pestis* antiserum after heating to 100 C for 30 minutes. In a few strains the common antigen was destroyed by boiling. With many strains the suspension agglutinated spontaneously after heating. There was no relation between the presence of heat stable antigen and susceptibility to lysis.

Antibodies were adsorbed from *P. pestis* antiserum with suspensions of *P. pseudotuberculosis* grown at 20 C and 37 C respectively and heated at 56 C for 30 minutes. The antigen grown at 37 C removed all antibodies for *P. pseudotuberculosis* grown at either 37 C or 20 C; the antigen grown at 20 C removed only antibodies for cultures grown at 20 C. These results and the differences in heat stability among various strains suggest that there may be more than one antigenic factor shared by the two species. Again there was no relation to lysis by P phage; antibodies were adsorbed from *P. pestis* antiserum as readily by a completely resistant strain as by an extremely sensitive one. Strains lysed by P phage were not demonstrably more closely related antigenically to *P. pestis* than were those not lysed.

The group and type specific somatic antigens of *P. pseudotuberculosis* were not related to sensitivity to phage.

Members of the various groups and types might or might not be lysed by P phage at 37 C or by PTB phage at 20 C. All strains but one were shown to possess the common flagellar antigen at 20 C but never at 37 C whether or not they were lysed.

An attempt was made to determine whether the adsorption of phage on cells of *P. pseudotuberculosis* would render them less agglutinable. Suspensions of *P. pseudotuberculosis* in 0.3% salt solution were heated at 56 C for 30 minutes and were then mixed with P phage or with PTB phage and incubated at 37 C over night. The phage titer of the supernatant fluid was determined before and after incubation. Most of the phage was removed from the supernatant fluid during incubation and presumably adsorbed on the cells. Phage incubated without bacteria showed no decrease in activity. Using the suspensions as antigen, agglutination tests were then performed with *P. pestis* and *P. pseudotuberculosis* antiserums. There was no change in agglutinability of the bacteria following adsorption of phage, suggesting that the "receptors" for phage and for antibodies may not be identical.

DISCUSSION

With many species of bacteria there is a close relationship between the antigenic components of the cell surface and susceptibility to lysis by phage, but antigenic structure and sensitivity to phage are not completely parallel. Evidence of this relationship has been summarized by Burnet (1930) and by Topley and Wilson (1946). Burnet (1929, 1930) found that although organisms with similar heat stable antigens show similar reactions toward bacteriophage, completely resistant variant strains may be serologically identical with the sensitive parent cultures. Furthermore, antigenically identical bacteria may fail to give the same reactions with a series of phages (Craigie and Yen, 1938).

Although all strains of *P. pseudotuberculosis* grown at 37 C share at least one antigenic component with *P. pestis*, they vary in their sensitivity to lysis by P phage. Those strains with a tendency to dissociate into the rough form are usually more sensitive to lysis than are smooth strains, suggesting that the loss of smooth surface antigen might unmask the components shared with *P. pestis* thus rendering the cell more susceptible to P phage.

The greater degree of lysis at 37 C than at 20 C may be associated with the tendency of most strains of *P. pseudotuberculosis* to produce rough variants at the higher temperature. It is also possible that the presence of a slime layer at 20 C might interfere with lysis by masking the components of the cell on which phage is adsorbed or by itself adsorbing phage before it reaches the cell surface. Bacterial polysaccharides are known to inhibit the lytic action of phage (Ashenburg et al, 1950). However, this hypothesis is inadequate because strains sensitive to phage seem to produce a slime layer as readily as do resistant cultures. Perhaps metabolic differences at the two temperatures might result in production of cofactors required for phage action at 37 C (Anderson, 1948) or of inhibiting factors at 20 C (Delbrück, 1948).

The location of the antigen shared with *P. pestis* within the cell of *P. pseudotuberculosis* is unknown, but at least one component probably lies near the surface and is readily extracted in 0.3% salt solution. Strains lysed by P phage might contain antigenic substances absent in resistant strains, but all attempts to demonstrate any clear

cut differences in the antigenic structure of sensitive and resistant strains were unsuccessful. Possibly qualitative or quantitative differences might be revealed in either the slime layer or the somatic surface by chemical extraction as used for *P. pestis* (Baker et al, 1947) or for *Shigella dysenteriae* (Morgan, 1936).

There is no apparent explanation for the differences in behavior of various strains of *P. pseudotuberculosis*. A few strains seem to be closely related to *P. pestis* as judged by their sensitivity to P phage at both 37 C and 20 C; others are intermediate, lysed at 37 C but not at 20 C; and a few seem unrelated and completely resistant to lysis. The similarity between certain strains of *P. pestis* and of *P. pseudotuberculosis* does not seem to justify placing these species in a new genus *Yersinia* as suggested by Van Loghem (1946) and Girard (1950).

No strain of *P. pseudotuberculosis* was lysed by high dilutions (10^{-5} to 10^{-6}) of P phage at 20 C. Hence, the use of a critical dilution of P phage at 20 C giving lysis of *P. pestis* but not of *P. pseudotuberculosis* should aid in rapid differentiation of the two species (Gunnison et al, 1950).

SUMMARY

A bacteriophage for *Pasteurella pestis* ("P phage") originally lysed 31 of 40 strains of *Pasteurella pseudotuberculosis* when tested at 37 C. After propagation of P phage on strains of *P. pseudotuberculosis* against which it had little action originally, it became adapted ("PTB phage") and lysed all 40 strains at 37 C. After propagation of P phage on strains of *P. pseudotuberculosis* which were highly sensitive originally, it showed no alteration in its behavior.

Following storage of the *P. pseudotuberculosis* cultures at 10 C for 6 months, all strains were still lysed at 37 C by PTB phage; but 16 strains were no longer lysed by P phage. At room temperature (20 C) following storage, only 20 strains were lysed by PTB phage and 8 by P phage. No strain of *P. pseudotuberculosis* was lysed by high dilutions (10^{-5}) of P phage at 20 C.

After repeated subculture at 37 C, *P. pseudotuberculosis* usually regained its sensitivity to P phage, but this was quickly lost again at 20 C. Repeated cultivation at 20 C tended to reduce the sensitivity to P phage.

No microscopic differences in morphology of sensitive and resistant strains of *P. pseudotuberculosis* could be demonstrated. All strains showed flagella at 20 C but not at 37 C. Sensitive strains produced a greater proportion of rough colonies than did resistant strains, especially at 37 C.

Strains of *P. pseudotuberculosis* which agglutinated spontaneously were more susceptible to lysis than strains which produced stable suspensions.

All stable suspensions of *P. pseudotuberculosis* cultivated at 37 C agglutinated with *P. pestis* antiserum; some strains cultivated at 20 C also agglutinated, but to lower titer. There was no correlation between agglutination with *P. pestis* antiserum and lysis by P phage. Strains lysed by P phage showed no closer antigenic relationship to *P. pestis* than did resistant strains.

The evidence presented indicates an absence of correlation between antigenic structure and sensitivity to phage.

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THE AUGMENTATION OF THE THERMOGENIC EFFECTS OF PYROGENS BY HOMOLOGOUS PLASMA IN RABBITS

VIRGIL S. LEQUIRE*

From the Naval Medical Research Institute, Bethesda, Maryland

The augmentation of the thermogenic and leukocytic effects of intravenously administered typhoid vaccine by homologous plasma in rabbits has been described in another report.[1] Ten times the number of typhoid organisms were required in a saline suspension to produce effects comparable to those obtained with a given number of the same organisms suspended in plasma. This augmentation was found in the two strains of *Eberthella typhosa* studied, and the onset of the response was earlier, following the injection of the plasma-typhoid suspensions.

Probey and Pittman[2] have reported a similar increase in the pyrogenicity of 28 cultures of viable organisms which had been grown in rabbit serum for 2 to 14 days. Mondolfo and Hounie[3] have shown an increase in the pyrogenicity of *Escherichia coli* organisms grown in blood serum as compared with those grown in unenriched broth.

The purpose of this study was to investigate the mechanism of this augmentation and to determine whether other pyrogens would be augmented in a similar manner.

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* Present address: Department of Anatomy, Vanderbilt School of Medicine, Nashville, Tenn.

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MATERIALS AND METHODS

Animals.—Male rabbits of mixed breed weighing 2500 to 3000 g were used. During experiments they were placed in individual stalls and secured by loose-fitting neckboards. Three rectal temperatures were taken at 30-minute intervals to establish a base line. The pyrogenic preparations were then injected into the marginal ear vein, and post injection readings were made each 30 minutes thereafter for 6 hours. Animals were given no food or water and were not removed from the stalls during experiments. A new group of animals was used in each experiment. Room temperature was maintained at 21 to 23 C.

Bacterial and virus preparations.—The pyrogenic materials employed in this study were: (a) strain 58 *E. typhosa*† containing approximately two billion organisms per ml and 0.05% phenol added as preservative; (b) strain 901 W *E. typhosa*, from the culture collection of the Naval Medical Research Institute. This organism was grown on tryptose phosphate agar at 37 C for 4 days, washed off with saline, then separated by centrifugation, suspended in isotonic saline, and killed by immersion in boiling water for 15 minutes. The density of the final suspension was approximately one billion organisms per ml; (c) purified *E. typhosa* pyrogen;[4]‡ (d) purified *Pseudomonas aeruginosa* pyrogen;[5]§ (e) purified *Aerobacter cloacae* pyrogen§ prepared by the method described by Welch;[5] (f) the egg-adapted PR-8 strain of influenza virus A.[6] All virus suspensions had a hemagglutinin titer adjusted to 1:1024 by dilution with apyrogenic isotonic saline.

An amount of pyrogen necessary to produce minimal to moderate febrile reactions was em-

† Obtained from the Army Medical Center, Washington, D. C.

‡ Obtained from Dr. Herbert R. Morgan, Department of Epidemiology, University of Michigan, School of Public Health, Ann Arbor, Mich.

§ Obtained from Mr. T. F. Probey, National Institutes of Health, Bethesda, Md.

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ployed in these experiments, since large amounts of pyrogen produced responses which obscured significant differences.[1]

Serology.—Agglutination titers were done on certain lots of immune and normal serum and plasma using strain 901 W *E. typhosa* as the antigen.[7]

The agglutination observed in these serums is not considered as indicative of the union of specific antibody with antigen, and therefore, will be referred to as clumping.

The technic for the production of human plasma as described by Newhouser and Kendrick[8] was used in preparing all plasma and serum employed in these experiments. The plasma used was prepared from whole blood to which sodium citrate (0.5%) had been added to prevent coagulation. Blood from three or more animals was pooled for each lot. Each lot of plasma and serum used was cultured and found to be sterile.

Preparation of materials to be injected.—A standardized method of preparing the plasma-pyrogen and saline-pyrogen suspensions was employed throughout the study. One ml of various dilutions of the stock pyrogens was added to vacuum tubes* containing 5 ml of plasma, serum or isotonic saline. For the controls, one ml of saline was added to 5 ml of plasma or serum. The tubes were agitated to assure thorough mixing and placed in a refrigerator at 5 C for 18 hours. Prior to injection, all solutions were warmed to room temperature.

The plasma, serum and isotonic saline used in each group of experiments were tested in animals and were not pyrogenic. Glassware, syringes and needles were sterilized by dry heat at 170 C for two hours. This is sufficient to inactivate any pyrogen present.[9]

EXPERIMENTAL RESULTS

In these experiments the temperature curves for individual animals in any given group were similar in magnitude and uniformity of response. Therefore, composite curves have been used to compare differences between groups. Using the mean temperature at the time of injection for the base line, average temperature changes were charted at 30-minute intervals for 6 hours. The temperature response of rabbits to intravenously injected apyrogenic saline and homologous plasma did not show significant differences from those of non-injected animals studied under similar experimental conditions.[1,10]

Augmentation of purified pyrogens and the pyrogenicity of a virus.—To test the hypothesis that other pyrogenic substances would be augmented by homologous plasma, as was suggested by the work of Probey[2] and Mondolfo,[3] three purified pyrogens and PR-8 strain of influenza virus A were studied. Purified pyrogens were employed in an attempt to determine if the bacterial cell is a necessary factor for the augmentation phenomenon.

The thermogenic response of rabbits to injections of purified pyrogens of *E. typhosa*, *Ps. aeruginosa*, and *A. cloacae* suspended in homologous plasma was significantly greater than was the response to the same pyrogens suspended in saline. Not only was the thermogenic response to the pyrogen-plasma suspensions definitely higher, but the thermal reaction was materially prolonged, and the latent period following injection was shortened as compared with results obtained with the pyrogen-saline suspensions (fig. 1–3).

The thermogenic responses induced by influenza virus A suspended in homologous plasma and in saline were approximately equal in deviation from the preinjection base, and both groups attained the maximum elevation at approximately the same time after

* Ten ml Vacutainers supplied by the Becton-Dickinson and Company, Rutherford, N. J.

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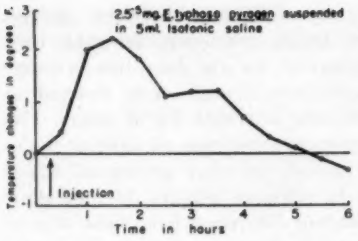


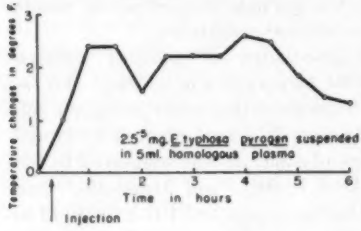
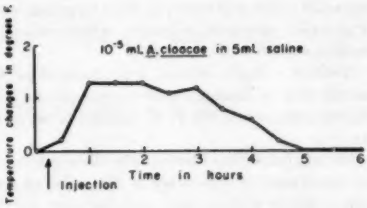


Figure 1.



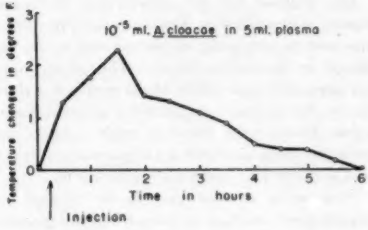
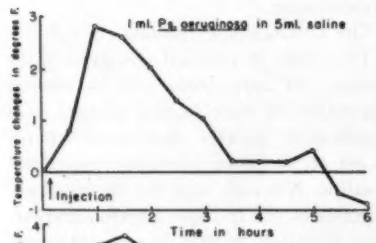


Figure 2.



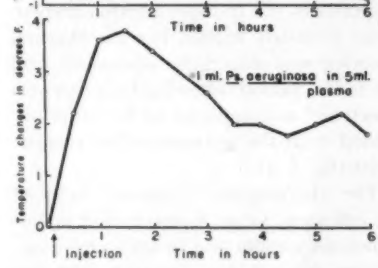
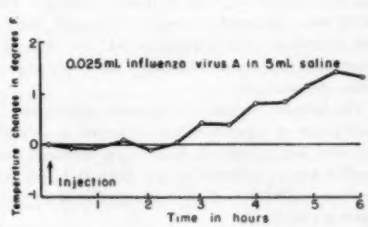


Figure 3.



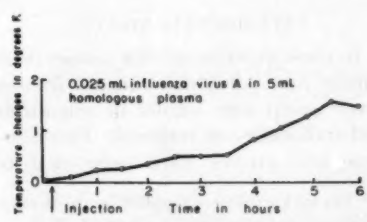


Figure 4.

FIG. 1.—Thermogenic response of rabbits (groups of 5 animals) to purified *E. typhosa* pyrogen suspended in isotonic saline and in homologous plasma.

FIG. 2.—The thermogenic response of rabbits (groups of 5 animals) to purified *A. cloacae* pyrogen suspended in isotonic saline and in homologous plasma.

FIG. 3.—The thermogenic response of rabbits (groups of 4 animals) to purified *Ps. aeruginosa* pyrogen (Welch) suspended in isotonic saline and in homologous plasma.

FIG. 4.—The thermogenic response of rabbits (groups of 10 animals) to the PR-8 strain of influenza virus A (hemagglutinin titer 1:1024) suspended in isotonic saline and in homologous plasma.

injection. However, there was a difference in the type of reaction induced by the two suspensions. Following injection with the virus-plasma suspensions, the latent period was quite short, 30 minutes as compared with 150 minutes, and the time period of fever was more prolonged, 5½ hours as compared with 3½ hours. The thermogenic reaction to influenza virus A is augmented with reimmune and nonimmune plasma and serum.

Removal of bacteria from the circulation.—The work of Beeson[9] has indicated that the thermogenic response of animals to certain pyrogenic bacteria is a function of the rate at which the pyrogen is removed from the circulating blood. To test the hypothesis that the augmentation of killed *E. typhosa* or-

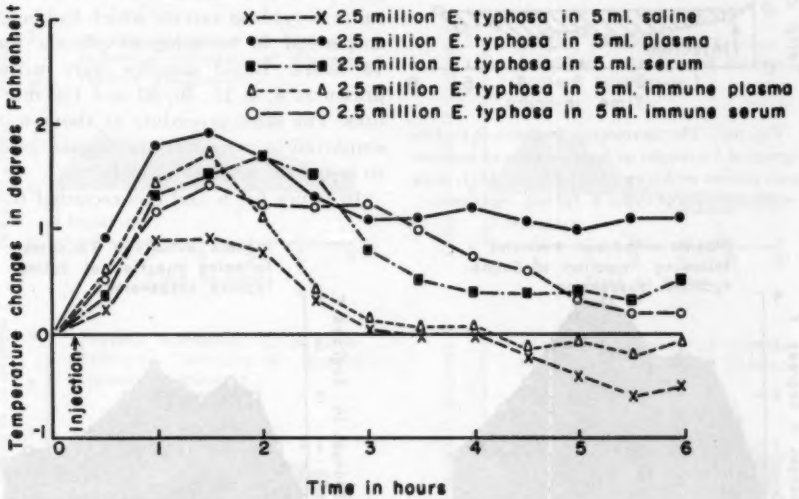


FIG. 5.—A graphic comparison of the thermogenic responses in rabbits (groups of 5 animals) to killed *E. typhosa* organisms (strain 901W) suspended in isotonic saline, in homologous nonimmune plasma and serum, and in homologous immune plasma and serum.

spect to the period of fever by suspension in homologous plasma (fig. 4).

The effect of immune plasma and serum upon the thermogenic response to E. typhosa.—The thermogenic response of rabbits to *E. typhosa* organisms suspended in immune plasma, immune serum, normal plasma and normal serum as compared with the response to the same number of organisms suspended in saline is shown in figure 5. These results demonstrate that the thermogenic response is augmented by both plasma and serum and by both

ganisms by homologous plasma might be related to this, the following experiment was designed.

Twenty rabbits were injected intravenously with 500 million killed *E. typhosa* organisms (strain 58) per kg body weight suspended in 5 ml isotonic saline, at the rate of 1 ml in 5 seconds. Animals were prepared for cardiac puncture, and 8 ml samples of blood were withdrawn into syringes containing 2 ml of 2.5% sodium citrate at exactly 4, 8, 15 and 30 minutes after the beginning of injection. Samples were

also withdrawn before injection of the typhoid vaccine to serve as controls. Not more than three samples were taken from any one animal. The samples of blood were put into vacuum tubes and the cells removed by centrifugation. The plasma was withdrawn with a syringe and injected into another vacuum tube. The plasma samples were then put in a refrigerator at 5 C for 18 hours. Five ml of each plasma sample was injected intravenously into normal rabbits.

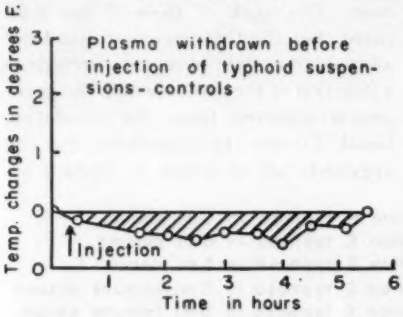


FIG. 6a.—The thermogenic response of rabbits (group of 5 animals) to 5 ml samples of homologous plasma withdrawn from donor rabbits prior to the injection of killed *E. typhosa* organisms.

Another group of 20 rabbits was injected with a similar amount of the same *E. typhosa* vaccine which had been suspended in homologous plasma for 18 hours. Blood samples were withdrawn at 4, 8, 15, 30, 60 and 120 minutes. The same procedure as above was employed in preparing the plasma used to inject the normal animals.

In figure 6a, b and c is recorded the

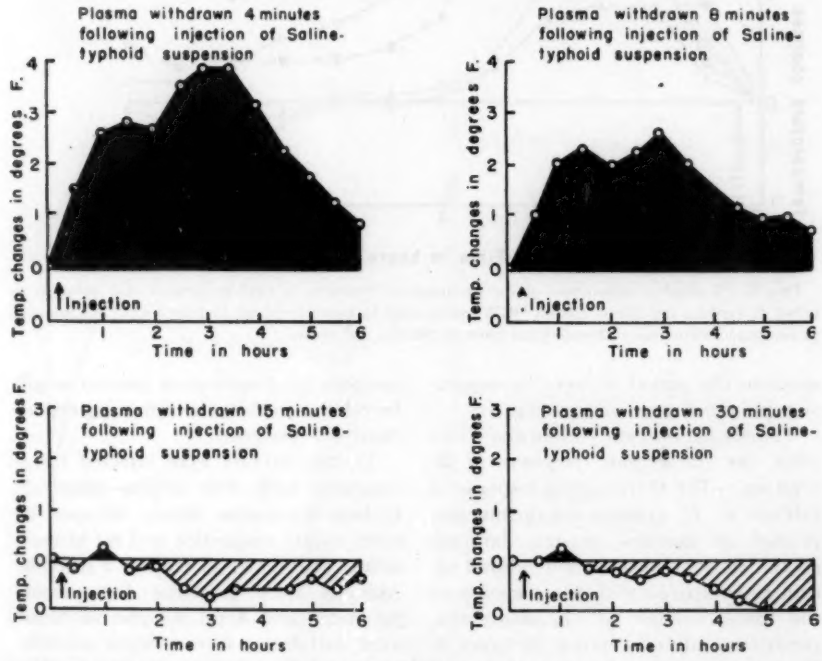

Plasma withdrawn 4 minutes following injection of Saline-typhoid suspension
Plasma withdrawn 8 minutes following injection of Saline-typhoid suspension
Plasma withdrawn 15 minutes following injection of Saline-typhoid suspension
Plasma withdrawn 30 minutes following injection of Saline-typhoid suspension
Temp. changes in degrees F.
Injection
Time in hours

FIG. 6b.—The thermogenic responses in rabbits (groups of 5 animals) to 5 ml samples of homologous plasma, withdrawn at various time intervals from donor rabbits which had been injected with 500 million killed *E. typhosa* organisms (strain 58) per kg body weight suspended in 5 ml isotonic saline.

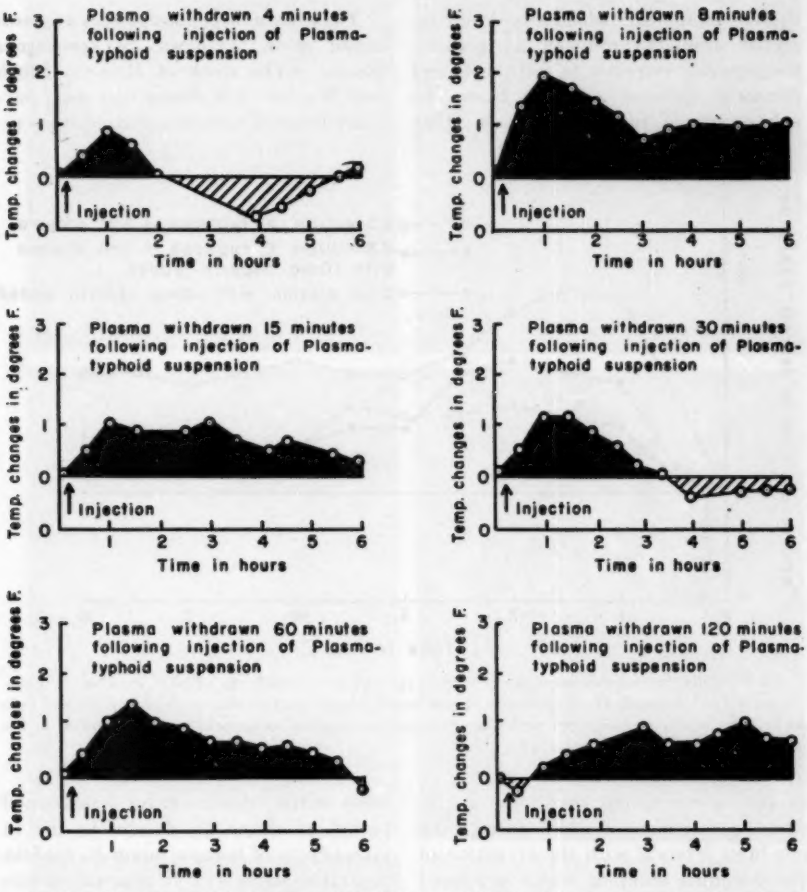

Plasma withdrawn 4 minutes following injection of Plasma-typhoid suspension
Plasma withdrawn 8 minutes following injection of Plasma-typhoid suspension
Plasma withdrawn 15 minutes following injection of Plasma-typhoid suspension
Plasma withdrawn 30 minutes following injection of Plasma-typhoid suspension
Plasma withdrawn 60 minutes following injection of Plasma-typhoid suspension
Plasma withdrawn 120 minutes following injection of Plasma-typhoid suspension
Temp. changes in degrees F.
Injection
Time in hours

FIG. 6c.—The thermogenic responses in rabbits (groups of 5 animals) to 5 ml samples of homologous plasma withdrawn at various time intervals from donor rabbits which had been injected with 500 million killed *E. typhosa* organisms (strain 58) per kg body weight suspended in 5 ml homologous plasma.

thermogenic response of normal rabbits injected with 5 ml of plasma withdrawn from donor animals at various time intervals. With this method of detecting pyrogenicity, it was evident that the donor animals injected with *E. typhosa* vaccine suspended in saline had removed all detectable amounts of pyrogen from the blood stream within 15 minutes following injection (fig. 6b). On the other hand, the plasma samples withdrawn from donor animals injected with the plasma-typhoid suspensions produced fever in animals through the two-hour time interval following injection (fig. 6c).

Another interesting observation was that none of the plasma samples with-

drawn from the plasma-typhoid injected animals produced as great a thermogenic response as did the 4- and 8-minute plasma samples from the saline-typhoid injected animals. Also, in the former group there was an increasing thermogenic effect through the one-hour interval with the exception of the 8-minute samples which produced the greatest response.

Control plasma samples were nonpyrogenic (fig. 6a), and samples withdrawn at 60 and 120 minutes from the saline-typhoid injected animals were nonpyrogenic.

The effect of heparin upon the augmentation of E. typhosa *by homologous plasma.*—The work of Knisely, Bloch and Warner[11] has shown that inert particles injected into heparinized frogs remain in the circulation for a prolonged period of time. To determine the in vivo effects of heparin upon *E. typhosa* organisms suspended in plasma, rabbits were injected with 5 ml suspensions of plasma to which 1.0 mg amounts of

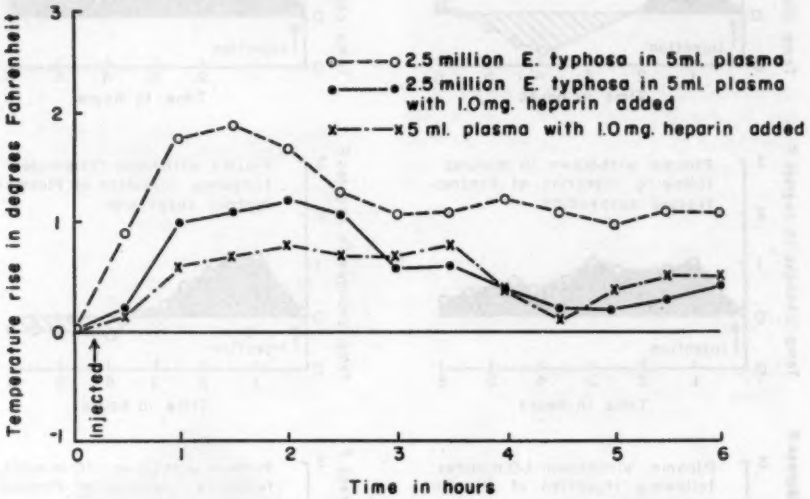
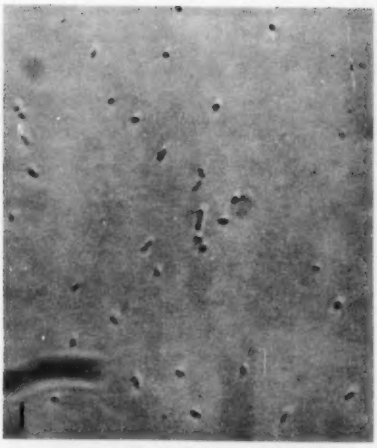


FIG. 7.—The thermogenic responses of rabbits (groups of 5 animals) to killed *E. typhosa* organism (strain 901 W) suspended in homologous plasma and in homologous plasma to which heparin had been added previously, as compared with the thermogenic response to heparin alone suspended in homologous plasma.

11. Knisely, M. H., Bloch, E. W. and Warner, L. 1948, Det Knogelige Danske Videnskabernes Selskab, Biologiske Skrifter, Bind IV, Nr. 7, Kobenhaven.

PLATE I

1.—Killed *E. typhosa* organisms suspended in saline. Phase microscope. ×1212.
2.—Killed *E. typhosa* organisms suspended in saline. Phase microscope. ×3031.
3.—Killed *E. typhosa* organisms clumped by normal rabbit plasma. Phase microscope. ×1212.
4.—Killed *E. typhosa* organisms clumped by normal rabbit plasma. Phase microscope. ×3031.
5.—Killed *E. typhosa* organisms coated in normal rabbit plasma. Phase microscope. ×1212.
6.—Killed *E. typhosa* organisms coated in normal rabbit plasma. Phase microscope. ×3031.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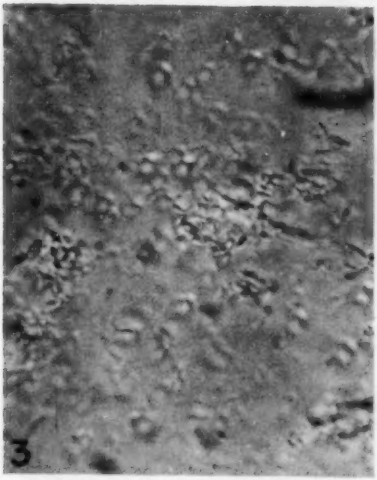
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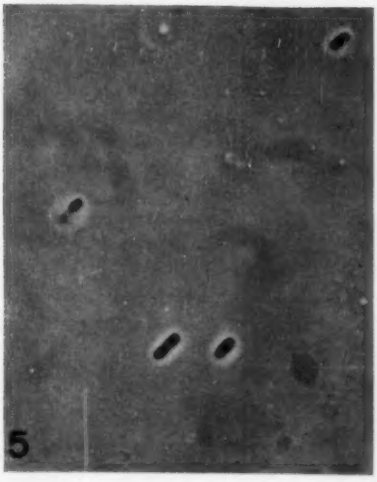
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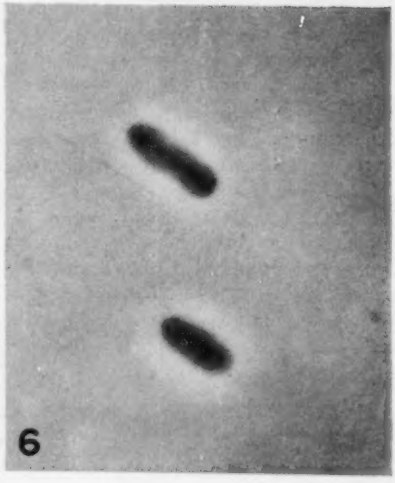
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Abbott's sodium heparin (10 mg will keep 5000 ml of plasma liquid in vitro at 37 C for 4 hours) had been added prior to the addition of *E. typhosa* or ganisms, strain 901 W. In comparing these results with those of animals re ceiving the same amounts of typhoid vaccine suspended in homologous plasma, it was found that the addition of heparin to the plasma-typhoid suspension decreased the thermogenic response and restored the latent period in onset of fever (fig. 7). It was also observed that heparin in the amounts used was pyrogenic, and the thermogenic response to plasma with heparin added was not significantly lower than the response to the heparinized plasma-typhoid suspension.

Observations of the in vitro changes that occur when bacteria and purified pyrogens are suspended in plasma and serum.—During the study of the mechanism of the augmentation of *E. typhosa* by homologous plasma, certain in vitro observations were made. Vaseline sealed coverslip preparations were studied under a Spencer phase microscope.

With dark phase under oil immersion, details of the bacteria were seen clearly. *E. typhosa* organisms which had been killed with heat consisted of dark granulations surrounded by a thin refractile line. There was no constant pattern in the distribution of these granulations within the bacterial cell. Structures resembling capsules were not observed (plate I; no. 1 and 2). The same structures were observed with the electron microscope, and conform essentially to the observations of colon bacilli by Hedén and Wyckoff.[12]

Living cultures of *A. cloacae* and *Ps. aeruginosa* were also observed microscopically. These organisms displayed a diffuse dark refractiveness without granulation.

When killed typhoid organisms suspended in plasma from normal animals were observed microscopically, they were seen to be clumped in large masses (plate I; no. 3 and 4). This clumping occurred immediately following mixing and could be seen grossly on a clean coverslip. There was no apparent pattern to this clumping. After a few minutes, thin strands of a material resembling fibrin were seen to form in the preparation and these frequently could be observed to radiate from a platelet within a mass of bacteria. This precipitation was frequently observed grossly as the formation of a fragile clot in test tubes of plasma to which bacteria had been added.

When the plasma-bacteria preparation was allowed to stand several hours, individual bacteria were seen to be surrounded by a highly refractive, distinctly outlined coat of a homogenous material (plate I; no. 5 and 6). This coating gave the bacteria an appearance not unlike the pneumococcus with its capsule undergoing the Quellung reaction. The coating formation could be followed on a single bacterium from a very thin line, indistinguishable from the halo of light, to a dense envelope three times the width of the bacterium. Clumped bacteria in plasma were observed to be enmeshed in an amorphous material similar in refractiveness to the material coating single bacteria. Purified pyrogens, which are nonparticulate, also caused the precipitation of fibrin strands and masses of amorphous material in plasma as observed grossly and microscopically.

Fresh normal serum clumped typhoid bacteria in a manner similar to that occurring in plasma, but the masses found were smaller im size, and strands of fibrin were infrequently seen. The thick coat-

12. Hedén, C. G. and Wyckoff, R. W. G. 1949 J. Bact. **58:** 153–160.

ing as observed in plasma was not seen in serum.

Serum from which a major part of the fibrinogen had been removed by thrombin clumped the bacteria, but this clumping took place over a period of hours and was of a different type than that seen in plasma. The clumps in serum with thrombin added were small with only a few bacteria sticking together, while the clumps in plasma ranged from 10 to 30μ and larger in diameter. In this serum, the formation of fibrin strands and coating of single bacteria as observed in plasma was not seen.

To compare the degree and type of clumping in normal serum and plasma with that in immune serum and plasma, serial dilutions of these serums were mixed with *E. typhosa* organisms using the technique of determining antigen antibody titers described by Bennet et al.[7] To tubes containing serial dilutions of serums an equal volume of antigen was added, and the tests were read after standing at room temperature for 18 hours. All tests were read by reflected light against a dark background, and the last tube showing clumping of the antigen visible to the unaided eye after shaking was taken as the end point. Immune serum and plasma was obtained from rabbits receiving three weekly injections of typhoid vaccine subcutaneously and then bled 7 days following the last injection. The plasma and serum used represent pooled lots from three or more animals.

Clumping as observed in normal serum was fine and granular in type and was present through 1:20 in twofold dilutions starting with 1:10. The clumping observed in normal plasma was heavy and floccular in type and was present through the 1:80 dilution with pellicle formation through the 1:40 dilution.

The clumping in both immune serum and immune plasma was observed through the 1:320 dilutions; however, there was a difference in the type of clumping. With immune serum the clumping in the 3+ and 4+ tubes was a heavy granular precipitate which could be easily dispersed by agitation. The clumping in immune plasma was floccular in type, and there was frequently pellicle formation in the 3+ and 4+ reactions which could not be easily dispersed by agitation.

To determine if the coating and clumping were related to the formation of fibrin, 0.5 mg sodium heparin was added to the plasma of both immune and nonimmunized rabbits, and *E. typhosa* organisms were then added. This was repeated in the presence of thrombin (Sharp and Dohme, 1 ml contained not less than 50 units). Results were read both macroscopically and microscopically.

Clumping of *E. typhosa* was inhibited but not prevented in nonimmune serum to which thrombin had been added in excess. Coating was not observed in this serum. Clumping was delayed in plasma from nonimmunized rabbits to which heparin was added in large amounts. Clumping was also delayed in the plasma of nonimmunized animals to which both heparin and thrombin were added, as observed microscopically. The type of clumping was different in plasma and in serum. In general, the clumping observed in serum to which thrombin had been added was similar to that seen in heparinized plasma, i.e., heparin inhibited the formation of the large floccular masses of bacteria in plasma.

DISCUSSION

Previous experiments[1] and those described above show that the thermogenic response of rabbits to killed *Eberthella typhosa* organisms can be

augmented by homologous plasma. This effect may be extended to purified pyrogens including *E. typhosa*, *Ps. aeruginosa*, and *A. cloacae*. This would indicate that the bacterial cell is not a necessary factor for augmentation. The results obtained with the PR-8 strain of influenza virus A indicate that the pyrogenicity of this virus can be augmented also.

The observation that the latent period in the onset of fever is shortened when the pyrogens were suspended in plasma would add evidence to the hypothesis that pyrogens do not act by a direct effect upon the temperature regulation centers in the brain,[9,12] and that a reaction takes place between the pyrogen and the constituents of the blood.

Beeson's work[9] has indicated that the rate of removal of "bacterial toxins" from the blood stream is dependent upon the functional capacity of the reticulo-endothelial (R-E) system. He observed that repeated daily injection of animals with pyrogens increased the rate at which these toxins were removed from the circulating blood; blockade of the R-E system with thorotrast at this stage of tolerance lowered the rate at which the pyrogens were removed.

In our experiments it was found that suspending *E. typhosa* organisms in plasma for 18 hours before injection prolonged the clearance time of the bacterial pyrogen from the blood stream of normal rabbits. The plasma of animals injected with saline-typhoid suspensions was found to be nonpyrogenic 15 minutes following injection, while that from animals injected with plasma-typhoid suspensions was pyrogenic throughout a two-hour time interval. This would indicate that homologous plasma alters the bacterial pyrogen in some manner which prevents its being removed from the circulation rapidly by the R-E system.

Phagocytosis of bacteria and inert particles by the R-E system must be preceded by an alteration in the surface properties of the bacteria or particulate matter. The mechanism for the removal of bacteria from the blood stream may be considered similar to that which frees the circulation from inert particles of the same order of size.[17] Globulin fractions in immune serums deposit upon the surface of antigens, altering their surface properties, promoting agglutination and phagocytosis,[14,15] and Mudd[16] has reported that fresh normal serums modify the surface properties of acid-fast bacteria in a manner quantitatively less but not observably different qualitatively from the effects of immune serums.

Knisely et al[11] have shown that inert particles injected into the blood stream are coated with a glassy film of protein having the characteristics of fibrin. They observed that these particles stuck together in aggregates and that the coating could be inhibited by massive doses of heparin. They also observed that this coating was necessary before the particles could be phagocytosed and that the rate of removal of the particles from the circulation was dependent upon the speed at which the coating formed.

In our experiments individual typhoid bacilli were seen to be coated in

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plasma by a substance having a refractive index approximately that of the bacterial cell. This heavy coating was not observed in heparinized plasma and defibrinogenated serum. It was also noted that strands of a substance resembling fibrin were formed in plasma to which bacteria and purified pyrogens had been added. This was observed both grossly and microscopically. Platelets were frequently seen enmeshed in the masses of bacteria in plasma.

The observation was made that serum from which fibrinogen had been removed by thrombin did not clump the bacteria into large masses. There was clumping in this serum after an interval of hours characterized by only a few bacteria sticking together. Heparin in vitro prevented the formation of large clumps of bacteria in plasma and delayed the onset of the clumping observed. This was observed grossly as a change from floccular to granular clumping; microscopically the clumping in heparinized plasma could not be distinguished from that observed in defibrinogenated serum.

The sticking together of bacteria as observed in the various serums employed in these studies, as contrasted to the comparative dispersion of the bacteria in saline, is assumed to indicate an alteration in the surface properties of these bacteria by constituents of the serums employed. It is also assumed that this alteration is a preliminary step before phagocytosis of the pyrogens can occur but could possibly proceed beyond the optimum for phagocytosis.

If it is assumed that the coating of bacteria is preliminary to phagocytosis, our evidence that bacterial pyrogens suspended in plasma remain in the circulation longer would seem to be at variance with the above reports. Also, the in vitro coating of *E. typhosa* organisms as observed by us can not be assumed to be identical with that formed on inert particles as observed by Knisely and co-workers. However, the size of the aggregates formed as observed with the in vitro mixing of bacteria and plasma is such that individual phagocytes could not remove these masses from the blood stream immediately and their pyrogenic effects would therefore be greater.

Nonparticulate or purified pyrogenic extracts of bacteria and influenza virus A caused the precipitation of an amorphous material in plasma which had an appearance not unlike that material formed around bacteria which had been suspended in plasma. This precipitation was not observed in defibrinogenated serum and in heparinized plasma. It is therefore assumed that these pyrogens are incorporated into this amorphous material and that their removal from the circulation is dependent upon particulate size as in the case of the bacterial aggregates.

It is of interest that immune plasma and serum augment the thermogenic response of rabbits to typhoid vaccine. It has been demonstrated in the case of influenza virus A, that the pyrogenicity as well as the leukocyte effects were abolished by immune serum.[6] This suggests not only a difference in the two pyrogens but that factors other than the alteration in surface properties of the antigen play a part in the augmentation phenomenon.

The augmented thermogenic effects of rabbits to pyrogens suspended in normal plasma, normal serum, immune plasma and immune serum are apparently due to their being incorporated into particulate size too large to be removed from the circulation immediately. This assumption is supported by the evidence that massive clumping is prevented in heparinized plasma as observed in vitro and that the augmented thermogenic effect in vivo was abolished

when plasma was heparinized before bacteria were added.

SUMMARY AND CONCLUSIONS

The thermogenic effects of the purified pyrogens of *Eberthella typhosa*, *Aerobacter cloacae* and *Pseudomonas aeruginosa* were augmented by homologous plasma in rabbits. There is evidence also that the pyrogenicity of the PR-8 strain of influenza virus A is enhanced by plasma.

Immune plasma and serum as well as nonimmune plasma and serum augmented the thermogenic response of rabbits to *E. typhosa* organisms.

The latent period in the onset of fever was shortened with the injection of pyrogens suspended in plasma.

The pyrogenicity of the plasma from animals injected with *E. typhosa* vaccine persisted longer when the bacteria had been suspended in homologous plasma than when suspended in isotonic saline.

E. typhosa organisms were observed grossly and microscopically to form large aggregates when mixed with plasma; coating of the organisms was observed microscopically in vitro. The substance responsible for the clumping and coating had some of the characteristics of fibrin.

Heparin inhibited the coating and the formation of large aggregates of bacteria in plasma in vitro, and prevented the augmented thermogenic response to *E. typhosa* organisms suspended in plasma in vivo.

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